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PRINCIPAL INVESTIGATOR: Mien-Chie Hung, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
Smithville, Texas 78957

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas
Smithville, Texas 78957

E-Mail: mhung@mdanderson.org

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13. ABSTRACT (Maximum 200 Words)

During the funding period, we have completed the most Aims and subaims proposed in the proposal, i.e. to determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells; to understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer; and to test the anti-tumor activity of p202 in prostate cancer cells using preclincal gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model. In an attempt to initiate a p202 clinical trial, we have communicated with the FDA and been informed that the human gene is preferable over murine one. Therefore, we have proceeded to clone a human counterpart of p202 gene, the AIM2 (absent in melanoma). We have obtained encouraging results on AIM2 that has the similar effect of p202 in anti-growth activity in prostate cancer cells in cell culture, and exhibits an anti-tumor growth activity in animals bearing mammary tumors. To further extend the specificity for both androgen receptor-positive and –refractory prostate cancers, we have newly developed a broad spectrum of prostate cancer-specific promoter. We intend to test the therapeutic efficacy and specificity by using the broad spectrum of prostate cancer-specific promoter to drive the therapeutic gene, AIM2 in animals. In an attempt to discover more novel strategies to treat prostate cancer, we have found a natural compound, emodin, which has shown a promising killing effect on prostate cancer cells. To further pursue the potential application of AIM2 gene for prostate cancer in a gene therapy setting, we have submitted a grant proposal entitled "The AIM 2 Gene as a Tumor Suppressor in Prostate Cancer Cells" for the IDEA Award to the Department of Defense on February 10, 2004 deadline.

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INTRODUCTION:

The interferon family (IFN) is composed of three classes:α, β and γ (1). The IFN family not only plays an integral role in host defense system against certain tumors and foreign antigens such as viruses, bacteria and parasites; but also possesses immunomodulatory and cell growth-inhibitory activities. However, the molecular mechanisms involved in IFN's anti-tumor activity are remained elusive. In a recent study, several IFN-inducible proteins are implicated in the process of tumor suppression (2). Moreover, based on DNA analysis, 19 out of 95 IFN-inducible genes are differentially downregulated during prostate tumor progression (3). The anti-cellular function of IFNs has been attributed to their abilities to induce G_1 phase arrest in cell cycle (4-6). P202, an IFN-inducible gene is a primarily nuclear 52kd phosphoprotein, has been shown to have a growth retardation function that was presumably accomplished by its ability to bind several cell-cycle regulatory proteins such as E2Fs, AP1, NFκB and pRb, resulting in the failure of S phase entry (7-9). Using p202 as a therapeutic agent, we have demonstrated that the multiple anti-tumor activities in human cancer xenograft models including breast and pancreatic cancers (10-11). Tumor-bearing mice treated with liposome/p202 complex had suppression of tumor growth, inhibition of angiogenesis and metastasis. In an earlier study on human prostate cancer cells, we observed that augmented expression of p202 inhibits cellular proliferation and suppresses transformation phenotype *in vitro* (12). Our ultimate goal is to develop a gene therapy strategy that would specifically deliver p202 to the prostate cancer cells so that the "normal cells" would not be affected by such treatment. To accomplish our goal, three specific aims are proposed (see below). The success of those aims will constitute a scientific basis for p202-associated anti-tumor effect on prostate cancer cells and will enable us to develop a novel p202 gene therapy strategy against prostate cancer.

Within the three-year funding period, we have succeeded to complete most of the Aims (and subaims). In an attempt to develop the p202 gene as a therapeutic gene for human clinical trials, we initiated a communication with FDA and were told that the p202 gene is of mouse origin and it is not favorable for human clinical trials. Hence, to circumvent

the potential drawback of using murine p202 gene for clinical trials and to maintain the benefits of p202 therapeutic efficacy, we use the AIM2, a human IFN-inducible protein, as a potential therapeutic agent in place of p202. The structurally related human (AIM2, IFI16 and MNDA) and murine (p202, p203, p204 and D3) genes belong to the 200-family, a family consisting of IFN-inducible proteins (13-18).

BODY:

A. SPECIFIC AIMS: (NO CHANGES)

Specific Aim 1: To determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells.

a. determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells;

b. determine pro-apoptotic activity of p202 in response to therapeutic agents, e.g. TNFα.

Specific Aim 2: To understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer.

a. determine the effect of p202 on G1/S cell cycle regulators in prostate cancer cells;

b. determine the effect of p202 on G2/M cycle regulators in prostate cancer cells.

Specific Aim 3: To test the anti-tumor activity of p202 in prostate cancer cells using preclincal gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model.

a. test the anti-tumor activity of p202 gene for tumors induced by s.c. injection;

b. test the anti-tumor activities of p202 gene for orthotopic prostate model;

c. develop a prostate-specific gene therapy strategy.

B. STUDIES AND RESULTS:

Specific Aim 1: To determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells.

As reported last year, we have completed the **Specific Aim 1a**, the results, which indicated that p202 has anti-tumor and pro-apoptotic activities in prostate cancer cells,

are in press in *Molecular Carcinogenesis* (please see the APPENDIX 1). We have held preliminary discussions with the FDA regarding the feasibility of initiating a phase I clinical trial using the p202 gene. The initial response from the FDA is that a human gene is preferable because of the potential immunogenicity of a murine gene expressed at high levels. For this reason, we have initiated to identify human genes homologous to the mouse p202 and have now cloned by using RT-PCR two genes including the AIM2 (Absent in Melanoma) and NMDA (Myeloid Nuclear Differentiation Antigen). When those two genes were tested their ability to inhibit cell growth using colony formation assay, we have found that AIM2 but not NMDA possesses activity to inhibit breast cancer cells. Since AIM2 is of human origin, and there are obvious advantages to use a human gene in human clinical trials rather than a murine gene such as p202, therefore, we will explore AIM2's anti-tumor activity in prostate cancer cells and in animal models. So far, we have demonstrated that AIM2 has an anti-growth activity as p202 in both prostate and breast cancer cell lines (APPENDIX 2).

Regarding to the **Specific Aim 1b,** we have only observed that p202 has minimal response to TNF-α, a therapeutic agent in prostate cancer cells. However, to this end, we recently discovered an interesting compound, Emodin which is a nature product extracted from the roots of numerous plants of the genus Rhamnus and acts as a tyrosine kinase inhibitor, has a profound effect on prostate cancer cells' proliferation and survival. We have demonstrated that Emodin inhibits expression of androgen receptor and suppresses cell growth and induced apoptosis in prostate cancer cells (APPENDIX 3). Emodin prolongs the survival of C3(1)/SV40 transgenic mice attributed to downregulating AR and preventing disease progression, tumor invasion and maintaining body weight gain and physical activity. In addition, we have found a plausible mechanism for Emodin downregulating transcriptional activity of AR. We have submitted our findings to *Cancer Cell,* and obtained feedback from the reviewers. Currently, the manuscript is under revision.

Specific Aim 2: To understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor, and potential pro-apoptosis activities in prostate cancer.

Please see the APPENDIX 1, we have completed the both subaims proposed in Specific Aim2. Briefly, we have shown that (1) the active form of Rb (hypophosphorylated Rb) is involved in p202-mediated growth arrest, particularly in arresting in G_1 of cell cycle in prostate cancer cells; and (2) cyclin B and p55cdc, which are known to be essential for the G2/M transistion and for the normal metaphase-to-anaphase transition in mitotic stage, respectively, are downregulated in p202 expressed prostate cancer cells.

Specific Aim 3: To test the anti-tumor activity of p202 in prostate cancer cells using preclincal gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model.

As reported in the APPENDIX 1, we have completed three subaims proposed in the Specific Aim 3. However, as mentioned in Specific Aim 1, we have consulted with FDA regarding p202 in future clinical trials, they have indicated that a human gene is preferable to a murine gene. Our data suggest that the human AIM2 may fulfill this requirement.

To develop a prostate cancer gene therapy, the prostate cancer-specific promoters such as that of PSA (19-22), probasin (19,23) and hK2 (24) have been recently developed. However, these promoters harbor certain limitations for expression in prostate cancer cells. Moreover, their activities in prostate cancer cells are, in general, lower than those of commonly used non tissue-specific virus-based promoters such as cytomegalovirus (CMV) promoter. Several modified promoters such as the modified prostate-specific probasin gene promoter, ARR2PB have overcome this problem (23-25 and APPENDIX 1). As a matter of fact, the promoter activity of ARR2PB is even higher than that of CMV promoter in response to androgen stimulation. This promoter is highly active in androgen receptor (AR)-positive prostate cancer cell line such as LNCaP; but is completely inactive in the AR-negative prostate cancer cell lines such as PC3. **Therefore, the therapeutic application of ARR2PB promoter will be limited to the**

AR-positive prostate cancer only; and it cannot be applied to the AR-negative prostate cancer patients. Many androgen-independent prostate cancers, though are refractory to androgen, still have active AR (26-29). These tumors are androgen-independent, but they appear to remain AR dependent. In this regard, a promoter that can be enhanced by androgen/AR stimulation could be very useful for prostate cancer gene therapy as it could selectively enhance the expression of the therapeutic gene in prostate cancer cells including the androgen-dependent prostate cells as well as those androgen independent prostate cells that still contain active AR. To accomplish this goal--to develop a promoter that exhibits a broad prostate cancer specificity (i.e. the activity of promoter is higher in prostate cancer cells including both AR-positive and -negative prostate cancer cells than in other normal cells or tissues), the ARR2 element (androgen receptor responsive element 2) derived from the prostate cancer-specific promoter, probasin was fused to our newly adapted "chimeric", a two-step transcriptional amplification (TSTA) approach (30, 31). This promoter should also display a basal level activity in both AR-positive and -negative prostate cancer cells (i.e. the activity of promoter should be higher than or comparable to that of CMV promoter in prostate cancer cells). With this in mind, we made the following constructs (Figs. 5 and 6 of APPENDIX 2) using a human telomerase reverse transcriptase (hTERT) promoter that has been shown to possess certain specificities for cancer cells and used in cancer gene therapies including prostate cancer gene therapy (32-34). Our preliminary data suggest that a hTERTp-based vector with WPRE and ARR2 modification has comparable activity to CMV in both AR-positive and –negative prostate cancer cells, and this activity can be further increased in response to androgen stimulation in the AR-positive prostate cancer cells. Both ARR2.hTERTp-TSTA-Luc and ARR2.hTERTp-TSTA-Luc-WPRE are strong gene expressors in AR-positive prostate cells such as LNCaP (about 10 fold of CMV in response to androgen stimulation) (APPENDIX 2, Fig 6B upper panel) but are virtually silent in normal cells (APPENDIX 2, Fig 5D). Importantly, compared with a modified prostate cancer-specific promoter such as ARR2PB that is almost silent in AR-negative cells such as PC3 (Appendix 1), the ARR2.hTERTp-TSTA-Luc-WPRE construct holds the advantage that it still exhibits a strong activity (comparable to CMV promoter) in PC3 cells (APPENDIX 2, Fig. 6B lower panel). **The broad prostate**

cancer specificity and a high transcriptional activity of these vectors will be further investigated *in vitro* and *in vivo*

Based on those encouraging results, we have submitted a grant proposal entitled "The AIM 2 Gene as a Tumor Suppressor in Prostate Cancer Cells" for the Idea Award, Department of Defense Prostate Cancer Research Program on February 10, 2004 deadline (log number PC040914). In the proposal (APPENDIX 2) we intend to (1): determine the anti-tumor and the pro-apoptotic activities of the AIM2 in prostate cancer cells; (2): develop a broad prostate cancer-specific expression vector for gene therapy; and (3) test the anti-tumor activity of AIM2 in prostate cancer cells using preclinical gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer animal model.

KEY RESEARCH ACCOMPLISHMENTS:

SPECIFIC AIMS

- We have completed Specific Aim 1 a, Specific Aim 2 a to b, and Specific Aim 3 a to c.

AIM2 (APPENDIX 2)

- AIM 2 possesses an anti-growth activity as p202 has in both breast and prostate cancer cells, and suppress mammary tumor growth in animals.
- AIM2 expression, similar to p202, inhibits NF-κB-mediated transcription activation in response to TNFα, and represses p65 (NF-κB)-activated transcription.
- ARR2.hTERTp-TSTA-Luc-WPRE has broad specificity in AR-positive and –negative prostate cancer cell lines.

EMODIN (APPENDIX 3)

- Emodin inhibits prostate cancer cells proliferation especially AR-positive cells.
- Emodin inhibits AR transcriptional activity through preventing AR nuclear transloction.
- Emodin decreases association of AR and hsp90 and increases association of AR and MDM2, which in turn induces AR degradation through proteasome-mediated pathway in a ligand independent manner.
- Emodin prolongs the survival of C3(1)/SV40 transgenic mice attributed to downregulating AR and preventing disease progression, tumor invasion and maintaining body weight gain and physical activity.

REPORTABLE OUTCOMES:

1. Wen, Y., Giri, D., Yan, D.-H., Spohn, B., Zinner, R. G., Xia, W., Thompson, T.C., Matusik, R.J. and Hung, M.-C Prostate specific anti-tumor activity by probasin promoter-directed p202 expression. *Mol. Carcino.* 37:130-137, 2003.
2. Idea Award, Department of Defense Prostate Cancer Research Program, CDMRP/DOD, Log Number: PC040914 "The AIM2 Gene as a Tumor Suppressor in Prostate Cancer Cells", submitted for 02/10/04 deadline.
3. Cha, T.-L., Qiu, L., Wen, Y. and Hung, M.-C. Emodin downregulates androgen receptor and inhibits prostate cancer cell growth. *Cancer Cell* (*in revision*), 2004. (#CC-D-04-00978).

CONCLUSIONS:

In the last three years, we have completed most of the Specific Aims (and subaims, please see the Key Research Accomplishment section and APPENDIX 1). We currently examine the anti-growth activity of human AIM2 for prostate cancer in animal models and establish a broad spectrum of prostate cancer-specific promoter, which can drive AIM2, the therapeutic gene to treat prostate cancer. The initial results are encouraging (APPENDIX 2). Success of the pending grant proposal will constitute a scientific basis for developing a novel AIM2 gene therapy strategy against prostate cancer. In addition, we also found that Emodin could potentially be an useful therapeutic agent to treat prostate cancer (APPENDIX 3).

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APPENDICES

APPENDIX 1

Wen, Y., Giri, D., Yan, D.-H., Spohn, B., Zinner, R. G., Xia, W., Thompson, T.C., Matusik, R.J. and Hung, M.-C Prostate specific anti-tumor activity by probasin promoter-directed p202 expression. ***Mol. Carcino.*** **37:130-137, 2003.**

APPENIDX 2

Idea Award, Department of Defense Prostate Cancer Research Program, CDMRP/DOD, PC040914 "The AIM2 Gene as a Tumor Suppressor in Prostate Cancer Cells", submitted for 02/10/04 deadline.

APPENDIX 3

Cha, T.-L., Qiu, L., Wen, Y. and Hung, M.-C. Emodin downregulates androgen receptor and inhibits prostate cancer cell growth. ***Cancer Cell*** **(*in revision*), 2004. (#CC-D-04-00978).**

Appendix 1

MOLECULAR CARCINOGENESIS 37:130–137 (2003)

Prostate-Specific Antitumor Activity by Probasin Promoter-Directed p202 Expression

Yong Wen,[1] Dipak Giri,[1] Duen-Hwa Yan,[1] Bill Spohn,[1] Ralph G. Zinner,[2] Weiya Xia,[1] Timothy C. Thompson,[3] Robert J. Matusik,[4] and Mien-Chie Hung[1]*

[1]*Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas*
[2]*Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas*
[3]*Scott Department of Urology, Baylor College of Medicine, Veteran Affairs Medical Center, Houston, Texas*
[4]*Department of Urologic Surgery, Vanderbilt University, School of Medicine, Nashville, Tennessee*

p202, an interferon (IFN) inducible protein, arrests cell cycle at G_1 phase leading to cell growth retardation. We previously showed that ectopic expression of p202 in human prostate cancer cells renders growth inhibition and suppression of transformation phenotype in vitro. In this report, we showed that prostate cancer cells with stable expression of p202 were less tumorigenic than the parental cells. The antitumor activity of p202 was further demonstrated by an ex vivo treatment of prostate cancer cells with p202 expression vector that showed significant tumor suppression in mouse xenograft model. Importantly, to achieve a prostate-specific antitumor effect by p202, we employed a prostate-specific probasin (ARR_2PB) gene promoter to direct p202 expression (ARR_2PB-p202) in an androgen receptor (AR)–positive manner. The ARR_2PB-p202/liposome complex was systemically administered into mice bearing orthotopic AR-positive prostate tumors. We showed that parenteral administration of an ARR_2PB-p202/liposome preparation led to prostate-specific p202 expression and tumor suppression in orthotopic prostate cancer xenograft model. Furthermore, with DNA array technique, we showed that the expression of p202 was accompanied by downregulation of G_2/M phase cell-cycle regulators, cyclin B, and p55cdc. Together, our results suggest that p202 suppresses prostate tumor growth, and that a prostate-specific antitumor effect can be achieved by systemic administration of liposome-mediated delivery of ARR_2PB-p202. © 2003 Wiley-Liss, Inc.

Key words: p202; tumorigenecity; probasin; cyclin B; p55cdc

INTRODUCTION

The interferon (IFN) family of cytokines plays a crucial role in host defense system against viral, bacterial, and parasitic infections and certain tumors. In addition, they also possess immunomodulatory and cell growth-inhibitory activities. There are three classes of IFN: α, β, and γ [1]. The mechanism involved in tumor suppressor activity of IFNs has not been well established. However, several IFN-inducible proteins were implicated in the process of tumor suppression [2]. Consistent with that notion, a recent report based on DNA array analysis indicates that 19 of 95 differentially downregulated genes associated with prostate tumor progression are, in fact, IFN-inducible genes [3]. The anticellular function of IFNs has been attributed to their abilities to induce G_1 phase arrest in the cell cycle [4–6]. Human prostate cancer cells are also sensitive to the antimitotic action of IFNs [7,8]. Recent studies demonstrate the inhibitory effect of IFN-α on growth [9–11] and colony formation [8] in several human prostate carcinoma cell lines.

Besides the therapeutic effects of IFNs in certain clinical settings, there are also undesirable side effects, viz. fever, chills, anorexia, and anemia, associated with high-dose IFN, which is often required to obtain a therapeutic response [12,13]. This has impeded IFN as an effective anticancer agent. In an attempt to circumvent this disadvantage and to harvest the benefit of IFN treatment, we explored the possibility of using an IFN-inducible protein, p202 [14], as a potential therapeutic agent. p202 belongs to murine 200 amino-acid protein family. Although the physiological function of p202 is not well defined, the experimental evidence gathered so far suggests its role in cell-cycle control, differentiation, and apoptosis [15,16]. In particular,

Yong Wen, Dipak Giri, and Duen-Hwa Yan contributed equally to this work.

*Correspondence to: Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd. Houston, Texas 77030.

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Abbreviations: IFN, interferon; Rb, retinoblastoma; ARR_2PB, modified prostate–specific probasin gene promoter; Luc, luciferase; PEI, polyethylenimine; PBS, phosphate buffer saline; SN, a cationic liposome formulation; AR, androgen receptor.

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ectopic p202 expression in cells results in retardation of growth that is thought to be mediated by E2F/retinoblastoma (Rb) pathway leading to G_1 arrest [17,18].

With p202 as a therapeutic agent, we have demonstrated the multiple antitumor activities in human cancer xenograft models, including breast and pancreatic cancers [19,20]. Tumor-bearing mice treated with liposome/p202 complex had suppression of tumor growth, inhibition of angiogenesis, and metastasis. In an earlier study on human prostate cancer cells, we observed that augmented expression of p202 inhibits cellular proliferation and suppresses transformation phenotype in vitro [21]. However, it has not yet been determined whether p202 expression inhibits the tumorigenicity of prostate cancer cells and whether the p202-based gene therapy is feasible in human prostate cancer xenograft model. In this report, we showed that p202 expression reduced the tumorigenicity of prostate cancer cells. With modified prostate-specific probasin gene promoter (ARR_2PB)-p202, a p202 expression vector driven by ARR_2PB promoter [22–24], we showed prostate-specific tumor suppression by ARR_2PB-p202. p202 expression was accompanied by downregulation of G_2/M phase cell-cycle regulators, cyclin B, and p55cdc [25–27].

MATERIALS AND METHODS

Cell Lines and Plasmids

LNCaP, MCF-7, PC-3, and four p202-expressing PC-3 clones, i.e., p202-1, -2, -3, and -4 [21] were cultured in Dulbecco's modified Eagle's medium/F12 media supplemented with 10% fetal bovine serum. The p202 expression vector CMV-p202 [18] is driven by CMV promoter. To construct the ARR_2PB-luciferase (Luc) vector, the ARR_2PB promoter element (468 bp), in pBlueScript II SK+ vector [22], was ligated into the KpnI/Sac I site of the PGL3-enhancer vector (Promega, Madison, WI). The ARR_2PB-p202 was generated by replacing the *Luc* gene in the ARR_2PB-Luc with the p202 coding sequence obtained from CMV-p202 vector [18] by BamHI digest. The correct orientation was confirmed by unique restriction enzyme digestion.

Subcutaneous and Ex Vivo Tumorigenicity Assays

PC-3 vector control (pcDNA3-pool), p202-1, and p202-2 cells (1×10^6 each) in 200 μL of phosphate buffer saline (PBS) were injected subcutaneously in 4- to 5-wk-old nude mice (five mice/ten tumors/cell line) on both sides of the abdomen. Tumor sizes were measured with a caliper once a week and tumor volume was calculated with the formula Vol. = S × S × L/2, where S = the short length of the tumor in cm, and L = the long length of the tumor in cm. For ex vivo experiment: PC3 cells growing in 100 mm dishes were transfected with 10 μg of CMV-p202 DNA complexed with 22.5 μg of polyethylenimine (PEI) for 45 min. PC3 cells were mock transfected with either CMV-p202 alone or PEI alone. After transfection, the cells were washed and incubated for an additional 18 h in complete media. Cells were then trypsinized, washed in PBS, counted, and 1×10^6 cells were inoculated s.c. in two sites on the flanks of male nude mice. Tumor size was measured weekly and volume calculated.

Transfection and Luc Assays

Human prostate cancer cell lines LNCaP and PC3, and a human pancreatic cancer cell line (Panc-1), were used for the reporter assay. Cells (2×10^6) were plated into a 6-well plate the day before transfection. Using SN2 liposome as a gene delivery system, cells were transfected with 0.5 μg of ARR_2-PB-Luc plasmid or 0.5 μg of CMV-Luc plasmid. pRL-TK (0.05 μg) was cotransfected as an internal control. Cells were harvested 36 h after transfection. The Luc activity was determined with the dual Luc protocol (Promega) with a luminometer.

Immunohistochemistry

The avidin-biotin peroxidase complex technique used in this study was modified from that described previously [28]. Briefly, formalin-fixed tissue sections were deparaffinized and dehydrated in ascending grades of ethanol. The sections were treated with 0.05% trypsin for 15 min, blocked in 0.3% hydrogen peroxidase in methanol for 15 min followed by treatment with 1% (v/v) normal horse serum for 30 min. The slides were incubated overnight at 4°C with anti-p202 goat polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:25 dilution. After liberal washing with PBS, the slides were incubated with biotinylated rabbit antigoat IgG at 1:200 dilution in PBS for 60 min at room temperature. The slides were subsequently incubated for 45 min at room temperature with the avidin-biotin-peroxidase complex diluted 1:100 in PBS. The product of enzymatic reaction was visualized with 0.125% aminoethylcarbazole, which gives a red colored reaction product. For counter staining, Mayer's hematoxylin was used.

Systemic Gene Therapy in Human Prostate Cancer Xenograft Model

Athymic nude mice (nu/nu) were opened through a single mid-ventral incision under sedation and the prostate gland was exposed. An aliquot of 30 μL of PBS containing 2×10^6 LNCaP cells were inoculated into the gland with a sterile syringe and 25 G needle. Such an inoculation resulted in a small swelling at the site. LNCaP cells under such conditions gave rise to tumors in 100% of animals as observed in a pilot experiment. The abdominal incision was closed with sterile stainless steel clips. A group of four animals was returned to a cage following recovery from the

sedation and recruited for the experiment. The treatment protocol was initiated 7 days after the intraprostatic inoculation of LNCaP cells, an interval sufficient to give rise to small tumors as observed in the pilot experiment. A dose of 25 μg of ARR_2PB-p202 plasmid DNA entrapped in a lipid formulation (SN) [29] at the ratio of 1:1.5 was incubated at room temperature for 30 min. The DNA/liposome complex was intravenously injected into the tail vein. The mice were treated twice a week for a period of 1½ months and then followed by treatment once a week. The Luc control group received an equivalent dose of plasmid DNA (ARR_2PB-Luc)/liposome complex. Animals were examined weekly to assess the tumor growth.

Western Blot Analysis

Protein lysate was prepared with RIPA-B cell lysis buffer containing 20 mM Na_2PO_4 (pH 7.4), 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 2 mM Na_3VO_4, 5 mM PMSF, 1% aprotinin, and 10 μg/mL of leupeptin. The antibodies specific for human Rb, cyclin B, p55cdc, and actin (Santa Cruz Biotechnology, Inc.) were used to detect these proteins by Western blot as described previously [19].

RESULTS AND DISCUSSION

p202 Suppressed Tumorigenicity of Prostate Cancer Cells

To investigate whether p202 could exhibit growth inhibitory effect on prostate tumor in vivo, two assays were performed. The first assay employed two p202 stable cell lines derived from human prostate cancer cell line, PC-3 [21]. The second was an ex vivo tumorigenicity assay with PC-3 cells transfected with p202. As shown in Figure 1A, 16 wks postimplantation, p202-1 and p202-2 clones generated smaller tumors than that of the control, the pcDNA3-pooled cell line. In fact, the p202-2 clone failed to form tumors in mice under identical experimental conditions. The difference in tumorigenesis between p202-1 and p202-2 may be attributed to an inadequate p202 protein expression in the former [21]. To rule out the possible contribution of clonal heterogeneity on the observed effects, we performed an ex vivo tumorigenicity assay in which PC-3 cells were transiently transfected with a p202 expression vector with a PEI vector delivery system. The transfected PC-3 cells were employed to generate subcutaneous xenografts in nude mice. The p202 transfected PC-3 cells, interestingly, showed no detectable tumor after 10 days (Figure 1B). On the contrary, the DNA control, i.e., CMV-p202 alone, was ineffective in containing tumor growth, indicating that the observed antitumor effect on PC-3 cells is attributable to p202 transfection. The vector controls, i.e., PEI alone, did not significantly affect tumor formation. Together, these results strongly suggested that p202 possesses an antitumor activity against prostate cancer cells. Importantly, it provides a scientific basis for developing a p202-based gene therapy strategy in an orthotopic human prostate cancer xenograft model.

ARR_2PB Promoter Directed Prostate-Specific p202 Expression and Tumor Suppression

To achieve prostate specific p202–mediated antitumor activity, we first tested whether an androgen receptor (AR)-responsive promoter could direct a Luc reporter gene expression in prostate cells. Because ARR_2PB promoter contains two copies of androgen response regions located upstream from a minimum PB promoter, it is highly responsive to androgen-dependent transcriptional activation [22]. We generated ARR_2PB-Luc and transfected it into two prostate cancer cell lines with (LNCaP) or without (PC-3)

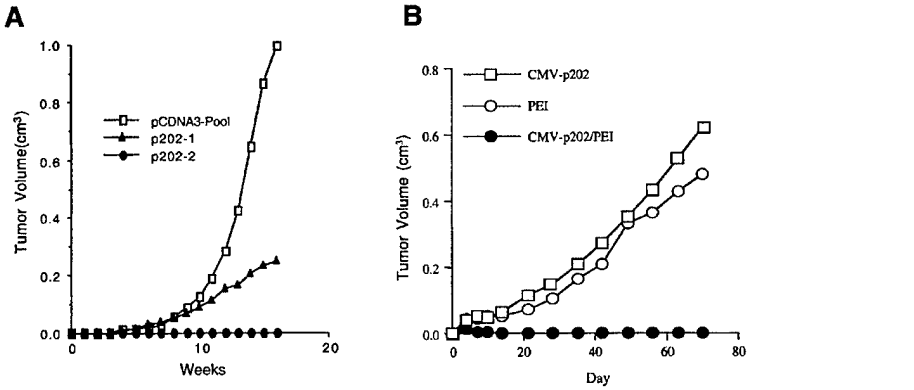

Figure 1. The antitumor function of p202 in PC-3 cells. (A) Reduced tumorigenicity of p202-expressing PC-3 cells. Nude mice (n = 5 per cell line) were injected subcutaneously with 1×10^6 cells in each of the two sides on the abdomen. Tumor size was measured each week and the tumor volume was calculated. The average tumor size at the indicated time points is presented. (B) p202 reduced the tumorigenicity of PC-3 cells ex vivo. PC-cells were transfected with CMV-p202/polyethylenimine (PEI) complex, PEI alone, or CMV-p202 alone. Eighteen hours after transfection, 1×10^6 cells were s.c. injected in both sides on the abdomen of a nude mouse.

endogenous AR expression. We used a pancreatic cancer cell line, Panc-1, as a nonprostatic control cell. As shown in Figure 2A, the relative Luc activity of ARR_2PB-Luc/CMV-Luc (a Luc gene expression vector driven by a constitutively active CMV promoter) was the highest in the AR-positive LNCaP cells, but not in AR-negative PC-3 and Panc-1 cells. This result suggested that ARR_2PB promoter activity was indeed AR-dependent [22], and thus confirmed the utility of ARR_2PB promoter to direct AR-specific gene expression in prostate cancer cells [23,24].

To test the AR-specific p202-mediated antitumor activity in orthotopic prostate cancer xenograft model, we generated a p202 expression vector driven by ARR_2PB promoter (ARR_2PB-p202). Likewise, ARR_2PB-Luc served as a negative control. The orthotopic prostate cancer xenograft model was established according to the procedure described previously [30,31]. After initiation of treatment 7 days after orthotopic tumor cell implantation in the prostate, survival time was prolonged in mice treated by ARR_2PB-p202. All mice treated with ARR_2PB-Luc were sacrificed on the 108th day post-treatment because they carried massive tumors and had reached the institutionally permissible limit for tumor burden (Figure 2B). In contrast, 100% of ARR_2PB-p202–treated mice were alive and healthy. Sixty percent (three mice) of the ARR_2PB-p202–treated mice survived on the 150th day post-treatment. To assess the antitumor activity, in an interim sacrifice protocol, three mice each from ARR_2PB-p202 and ARR_2PB-Luc treatment groups were euthanized and prostate glands dissected at day 77 of treatment. ARR_2PB-p202–treated tumors were remarkably reduced in size compared those treated by the control vector, ARR_2PB-Luc (Figure 2C). This observation explains the prolonged survival seen in mice treated by ARR_2PB-p202. The use of ARR_2PB promoter to direct expression of p202 predicts the specificity of effect. Therefore, we examined the p202 expression on tumors and organs isolated from ARR_2PB-p202–treated mice by immunohistochemical staining. The p202 protein was detected in the cytoplasm as a red colored reaction product from the enzymatic reaction with aminoethylcarbazole as the chromogen. Note abundant intracytoplasmic expression of p202 in the tumor from the mouse treated with ARR_2PB-p202 (Figure 3, left panel). The mouse treated with ARR_2PB-Luc had undetectable p202 (Figure 3, right panel). Given that p202 is primarily a nuclear protein [32], the exact reason for the predominant cytoplasmic staining of p202 is not clear. However, it is probably due to the robust expression of p202 that causes accumulation of p202 in the cytoplasm. Alternatively, because the induced p202 localizes in the cytoplasm for 30–36 h after IFN treatment before translocated into the nucleus [32], it is likely that p202 could still remain in the cytoplasm 20-h post–ARR_2PB-p202 treatment. We also examined the expression of p202 in multiple organs such as lung, liver, kidney, and heart to ascertain the nonprostatic expression, if any. There was no extraprostatic expression of p202 except the reticuloendothelial cells of lung and liver from both ARR_2PB-p202 and ARR_2PB-Luc–treated mice (data not shown and Figure 3). The p202-positive mouse reticuloendothelial cells is probably the endogenous level of p202 expression, because all 200 amino-acid protein family members are expressed in hematopoietic cells [33]. Together, the results strongly suggested that systemic delivery of ARR_2PB-directed expression vector by SN liposome could result in prostate and AR-specific antitumor activity in prostate cancer.

ARR_2PB promoter–mediated therapeutic gene expression is primarily useful for targeting AR-positive prostate cancer, which makes up a significant portion of the prostate cancer patient population. Although AR-negative prostate cancer is insensitive to androgen, in many of these cases, AR is still active [34]. It is conceivable that ARR_2PB promoter could be activated in these androgen-independent prostate tumors. In addition, ARR_2PB promoter is also responsive to glucocorticoids that have been routinely used to improve the quality of life in prostate cancer patients who failed androgen deprivation therapy [22,35]. Thus, ARR_2PB-p202 could be potentially used to achieve a prostate-specific therapeutic effect on androgen-independent prostate cancer patients who are treated with glucocorticoids.

p202 Upregulated the Hypophosphorylated Rb and Downregulated Cyclin B and p55cdc

To investigate the underlying mechanisms of the p202-mediated growth inhibition and tumor suppression in prostate cancer cells, we set out to determine (1) if Rb phosphorylation was involved in p202-mediated growth arrest because IFN treatment increases the level of hypophosphorylated (active) form of Rb [36–38]; and (2) other regulatory genes responsible for the p202-mediated growth retardation and tumor suppression that can be identified by DNA array technology. To examine the effect on Rb phosphorylation by p202, we employed Western blotting with a Rb-specific antibody to analyze the phosphorylation status of Rb in both parental and p202-expressing prostate cancer cells. Figure 4A shows that the p202-expressing cells, i.e., p202-1, -2, and -3, exhibit an elevated level of hypophosphorylated form (faster migrating band) of Rb as compared to the control, i.e., pcDNA3-pooled, in which the hyperphosphorylated form (slower migrating band) of Rb is most prevalent. Thus, one possible mechanism by which p202 induces cell growth arrest in PC-3 is by enhancing the level of hypophosphorylated Rb. Presumably, the active Rb would then inhibit E2F transactivation function by forming an Rb/E2F complex. Thus, the E2F-mediated transcription of S-phase genes might be inhibited

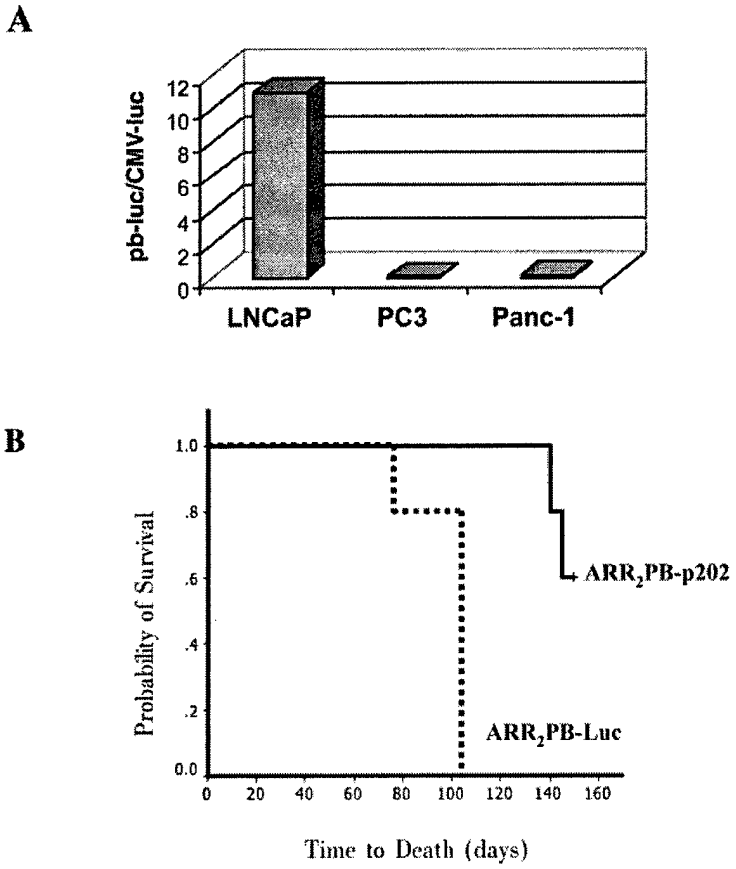

Figure 2. (A) Modified prostate-specific probasin gene promoter (ARR_2PB) activity was androgen receptor (AR)-dependent. ARR_2PB-luciferase (pb-Luc) (0.5 μg) and CMV-Luc (0.5 μg) were transfected into two prostate cancer cell lines with, for example, LNCaP, or without, for example, PC3, endogenous AR expression. A pancreatic cancer cell line, Panc-1, served as a nonprostatic cell control. The ratios of Luc activity resulted from ARR_2PB-Luc and CMV-Luc transfections were measured. pRL-TK (50 ng) was co-transfected and served as an internal control for transfection efficiency with dual Luc assay (Promega, Madison, WI). The data shown here are the average of two independent experiments. (B) Prolonged survival by ARR_2PB-p202 treatment. LNCaP orthotopic tumor-bearing mice ($n = 5$ per treatment group) were intravenously treated with ARR_2PB-p202 or ARR_2PB-Luc/SN liposome complexes. Survival rates were measured by Kaplan-Meier analysis. (C) Antitumor activity by systemic ARR_2PB-p202 treatment. Tumor suppression by ARR_2PB-p202 treatment. Representative LNCaP tumors ($n = 3$ per treatment group) are shown from mice treated with ARR_2PB-p202 or ARR_2PB-Luc/lipid formulation (SN) liposome complexes on day 77 post-treatment.

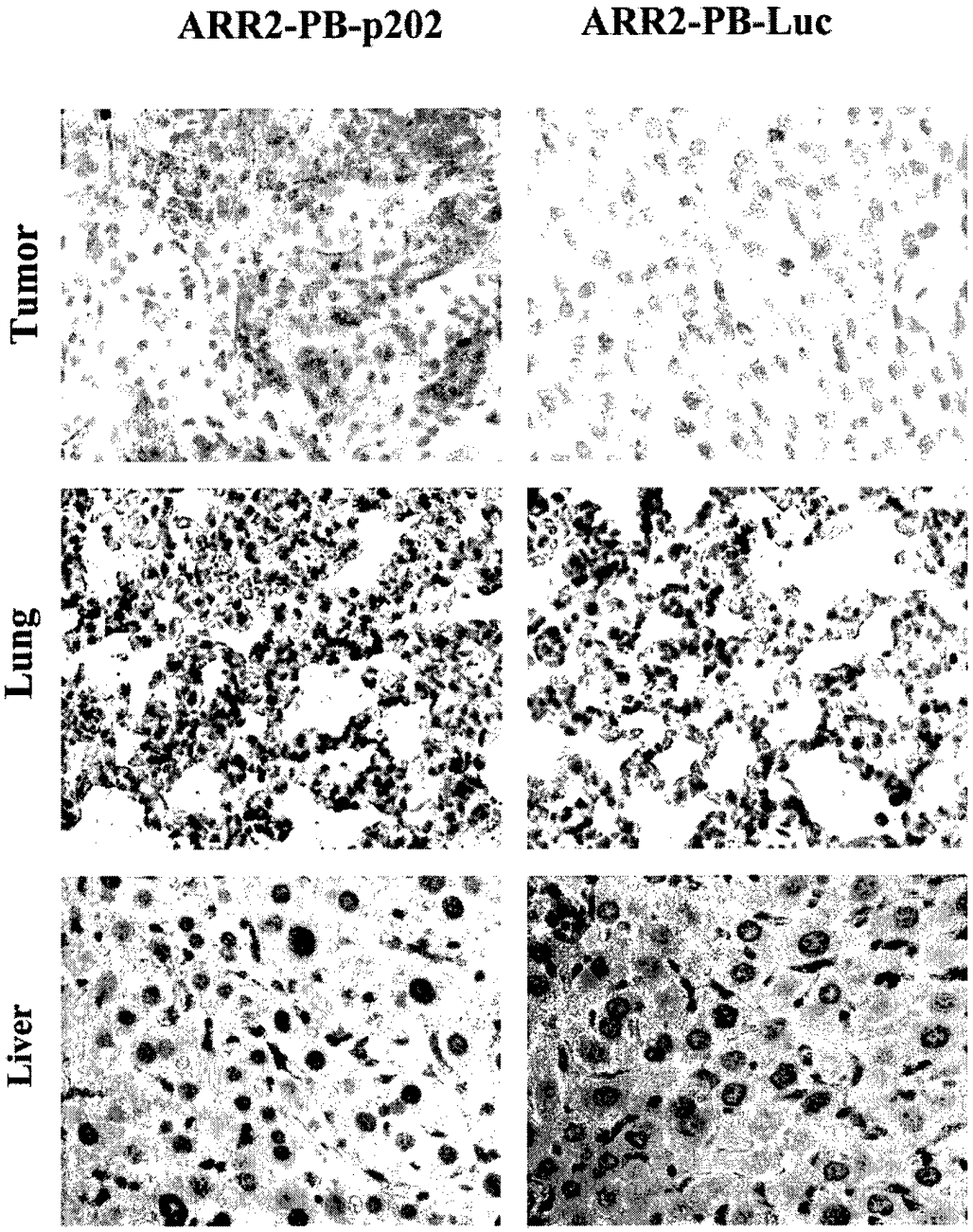

Figure 3. ARR_2PB promoter directed prostate-specific p202 expression. Formalin-fixed tumors, lung, and liver from mice 20-h post-treatment with ARR_2PB-p202 or ARR_2PB-Luc were sectioned and stained for p202 employing polyclonal anti–p202 antibodies as described in Materials and Methods.

causing G_1-phase arrest. Because p202 is a transcription modulator, it is possible that p202 could regulate certain gene expression which might be important in p202-mediated growth arrest and tumor suppression in prostate cancer cells. To identify other critical genes involved in p202-mediated antigrowth and antitumor activities, we employed DNA array technology. With RNA products obtained from PC-3 (parental control) and p202-2 (a representative p202-expressing prostate

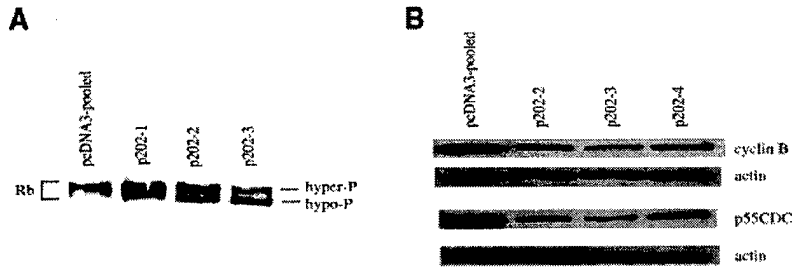

Figure 4. p202 enhanced hypophosphorylated retinoblastoma (Rb) and reduced cyclin B and p55CDC expression. Cell lysates obtained from pcDNA3-pool and p202-expressing PC-3 cell lines (p202-1, -2, -3, and -4) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently analyzed by Western blot with antibodies against Rb, cyclin B, p55cdc, and actin. The actin bands served as loading control.

cancer cell) to hybridize with DNA array filters (Clontech, Palo Alto, CA) containing 588 known genes that are involved in various cellular regulatory pathways including those of cell-cycle control, we were able to identify several candidate genes whose expression was found to be significantly influenced by p202 expression. Two such genes have been confirmed by Western blotting, i.e., cyclin B and p55cdc (Figure 4B), which showed a reduced level of expression in p202-expressing cells, as compared to the control, pcDNA3-pooled. The sample loading was similar, as indicated by the actin control. In light of the well-documented p202-mediated G_1 arrest, the reduction of cyclin B and p55cdc in p202-expressing cells is rather surprising, because cyclin B is involved in G_2/M phase transition [25] and p55cdc is required for normal metaphase-to-anaphase transition involved in late mitotic events [26,27]. It is likely that the downregulation of these two genes by p202 may contribute to the p202-mediated cell-cycle arrest. This is the first time that p202 has been implicated in involvement in G_2/M phase cell-cycle control. It is possible that the p202-associated cyclin B and p55cdc downregulation may contribute, in part, to the p202-mediated growth arrest.

In this report, we showed that p202 expression suppressed the tumorigenicity of prostate cancer cells. A subsequent ex vivo experiment with either CMV-p202/CMV-PEI complex also inhibited prostate cancer cell growth in a xenograft model. The utility of p202 as a potential therapeutic gene for prostate cancer treatment was demonstrated by the observation that prostate-specific antitumor activity can be achieved by systemically treating the prostate tumor-bearing mice with a p202 expression vector driven by a composite probasin promoter, ARR_2PB. Thus, in addition to local and systemic treatment of breast and pancreatic tumors, respectively, by using a p202 expression vector driven by a constitutively active promoter such as CMV promoter [19,20], our results suggested the feasibility of using a tissue-specific promoter to achieve p202-mediated antitumor activity in those cancer types as well. Experiments are underway to test that possibility. Given that p202 is involved in G_0/G_1 transition by targeting E2F/Rb pathway [16], it is interesting to note that G_2/M cell-cycle regulators such as cyclin B and p55cdc are downregulated by p202. Further analysis on the p202 effect on G_2/M transition by downregulation of cyclin B and p55cdc will shed light on how p202 inhibits cell proliferation.

ACKNOWLEDGMENTS

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Appendix 2

Title/Referral Page
No Page Limit

a. Proposal title (up to 160 characters)

THE AIM 2 GENE AS A TUMOR SUPPRESSOR IN PROSTATE CANCER CELLS

b. Proposal log number

PC040914

c. PI's full name (first, middle initial, last)

MIEN-CHIE HUNG

d. PI's institution

THE UNIVERSITY OF TEXAS M. D. ANDERSON CANCER CENTER

e. Award mechanism

IDEA DEVELOPMENT AWARD

f. Please indicate if this is a:

X NEW proposal

☐ DUPLICATE proposal to another FY04 CDMRP program

☐ RESUBMITTED proposal to this program

g. Keyword descriptive technical terms

AIM2, IFN-INDUCIBLE 200-FAMILY PROTEINS, PROSTATE CANCER-SPECIFIC EXPRESSION VECTORS, GENE THERAPY, NONVIRAL LIPOSOME DELIVERY SYSTEM

h. Conflicts of interest: Include the following information (no page limit)

Name	Institutional Affiliation(s)	Role(s) on Proposed Project or Perceived Conflicts of Interest
Mien-Chie Hung	**UTMDACC**	**Principal Investigator**
Xianghuo He	**UTMDACC**	**Project Investigator**
Xiaoming Xie	**UTMDACC**	**Research Scientist**
Chao-Kai Chou	**UTMDACC**	**Graduate Research Assistant**

Idea Development Award Proposal
Table of Contents/Checklist

Proposal Log Number: PC040914

Principal Investigator: Hung (*Last Name*) Mien-Chie (*First Name*) (*MI*)

Proposal Title: The AIM2 gene as a tumor suppressor in prostate cancer cells

Yes		Page Number
☐	Proposal Information	N/A
☐	Statement of Work (11,400 character limit)	N/A
☐	Technical Abstract (5,700 character limit)	N/A
☐	Public Abstract (5,700 character limit)	N/A
☐	Title/Referral Page (no page limit)	N/A
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☐	Proposal Resubmission (if applicable)	N/A
☐	Proposal Resubmission Statement (2-page limit)	N/A
☐	Previous submission summary statement (no page limit)	N/A
X	Main Body (10-page limit)	**3**
X	Abbreviations (1-page limit)	**13**
X	References (no page limit)	**14**
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X	Facilities/Equipment Description (no page limit)	**30**
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X	Publications and/or Patent Abstracts (5-document limit)	**31**
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☐	List of items included in this section	N/A
☐	Letters of support from any collaborating individuals or institutions	N/A
☐	Competitive Continuation (if applicable)	N/A
☐	Progress Statement (2-page limit)	N/A
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☐	Budget Information	N/A
☐	Detailed Cost Estimate (no page limit)	N/A
☐	Budget Justification (no page limit)	N/A
☐	Regulatory Documents (Certificate of Environmental Compliance and Principal Investigator Safety Program Assurance Form)	N/A
☐	US Army Medical Research Acquisition Activity Documents (Rate Agreement, Certifications and Assurances for Assistance Agreements, and Representatives for Assistance Agreements)	N/A

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Proposal Relevance Statement

Prostate cancer is one of the most deadly diseases inflicting American men today. The frequent recurrence and the subsequent metastasis of the disease after traditional surgical and/or hormone ablation treatment pose a serious thereat to the survival of prostate cancer patients. Therefore, it has been an urgent need to develop novel strategies against such a deadly disease. In this current proposal, we attempt to address that by proposing a novel gene therapy strategy against prostate cancer based on our currently findings that the AIM2, a human interferon-inducible gene, could inhibit the growth and tumorigenecity of prostate cancer cells.

Three Tasks will be carried out to accomplish our goals. Task 1, we will test the AIM2-mediated anti-tumor activity in an orthotopic prostate cancer model. We will also test the therapeutic efficacy of a combined AIM2/TNF-α treatment to determine the potential AIM2-mediated sensitization of prostate cancer cells to TNF-α-induced apoptosis. Task 2, we will develop and characterize prostate cancer-specific expression vectors. In order to construct prostate cancer-specific expression vectors that are highly expressed in prostate cancer cells, but low in normal cells, either the human telomerase reverse transcriptase (hTERT) based promoter-driven or probasin, a prostate-specific promoter, driven vectors will be developed. Task 3, we will develop an AIM2-based gene therapy strategy using liposome as delivery system to test the therapeutic potential of AIM2 in an orthotopic prostate mouse model. Moreover, to achieve a prostate cancer-specific AIM2 expression, we will employ prostate-specific promoters which we will develop to test the prostate cancer specific expression of AIM2 gene-driven by these promoters and examine their therapeutic efficacy.

Success of this project will constitute a scientific basis for developing a novel AIM2 gene therapy strategy against prostate cancer.

1. BACKGROUND AND PRELIMINARY RESULTS

1-1. p202, an IFN-α inducible mouse protein, has been demonstrated to have a therapeutic potential in human prostate cancer cells. The Interferon (IFN) family of cytokines plays a crucial role in host defense system against viral, bacterial and parasitic infections and certain tumors (1). In addition, they also possess immunomodulatory and cell growth-inhibitory activities (2-8). Apart from the therapeutic effects of IFNs in certain clinical settings, there were also undesirable side effects (e.g., fever, chills, anorexia, and anemia) associated with a high dose of IFN treatment, which is often required to obtain a significant response (9-11). This has hampered the use of IFNs as effective anti-cancer agents (please see later). In an attempt to circumvent this potential drawback and to maintain the benefit of IFN treatment, we propose in this study to use AIM2, a human IFN-inducible protein, as a potential therapeutic agent. The structurally related human (AIM2, IFI16 and MNDA) and murine (p202, p203, p204 and D3) genes (12) belong to the 200-family, a family consisting of IFN-inducible proteins. Among them, p202 has been well characterized and shown to have a growth inhibitory function (13 and Appendix 1 and 2). Furthermore, p202 could inhibit tumorigenicity *in vivo* either by a tumorigenicity assay using p202-expressing PC3 cell lines or in an *ex vivo* tumorigenicity assay using the parental PC3 cells (Appendix 2). In addition, it prolonged survival for LNCaP orthotopic tumor-bearing mice under a gene therapy setting through intravenous delivery of p202 gene complexed with liposome (Appendix 2). We have also demonstrated that p202 associates potent anti-cancer activities including suppression of tumorigenicity, metastasis and angiogenesis in orthotopic animal models for breast and pancreatic cancers (Appendix 3,4). Thus, the p202 gene, a murine member of IFN-inducible 200 family possesses a strong anti-cancer activity in multiple animal models.

In an attempt to develop the p202 gene as a therapeutic gene for human clinical trials, we initiated a communication with FDA and were told that the p202 gene is of mouse origin and it is not favorable for human clinical trials. Hence, we turn our attention to the human 200 family members in a hope to identify a human gene associates with a strong anti-cancer activity as p202 does. To this end, we have isolated human 200 family genes through RT-PCR including AIM2, IFI16 and MNDA. The initial screening of cell-killing activity among these three human genes, we found that growth arrest of AIM2 is much potent than the other two; and is comparable to that of the p202 gene (data not shown). Therefore, in the current proposal, we propose to further characterize the anti-cancer activity of AIM2 gene, and intend to develop a gene therapy protocol using AIM2 as a therapeutic gene that is driven by a newly developed prostate cancer-specific expressing vector (see later).

1-2. AIM2 possesses the anti-proliferation, anti-transformation, and anti-tumor activities in human breast cancer cells.

1-2-1. AIM2 inhibits cancer cells growth in monolyer and colony formation in soft agar. AIM2 is a 39kDa protein, originally identified by subtractive cDNA selection and shown that it is absent in melanoma (14). Recently it has been shown that AIM2 is one of the most frequently mutated genes caused by microsatellite instability (MSI) in colorectal tumors (15). The ectopic expression of AIM2 in murine embryo cells retarded proliferation (16), suggesting that the AIM2 is a potential tumor suppressor. In support of this concept, we have found that AIM2 protein is associated with growth arrest in both breast and prostate cancer cells in monolyer culture. Furthermore, AIM2 can inhibit anchorage-independent growth of breast cancer cells. Two different types of cancer cell lines were used for growth arrest assay, including a prostate cancer cell line, PC3; and breast cancer cell lines, MDA-MB-231, MDA-MB-435, MDA-MB-453 and MCF-7. An AIM2 expressing plasmid (CMV-Flag-AIM2) together with reporter plasmid (CMV-luc) was transfected into those cell lines. Using the apparent luciferase (luc) activity as an indicative of living cells, we showed that AIM2 expression caused overall cell growth inhibition in a dose-dependent manner (Fig. 1).

A. PC3 B.

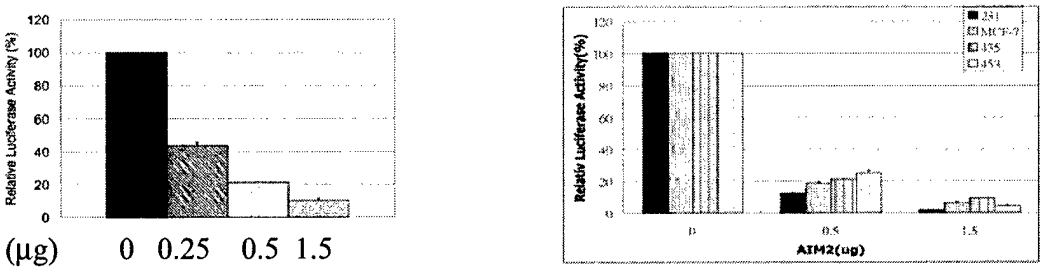


Fig.1. The Aim2 expressing cells exhibit a reduced growth *in vitro*. Different amount of the AIM2 expressing plasmid as indicated in each panel was transiently transfected into the indicated cell lines, i.e. prostate cancer cell line, PC3 (A), and different breast cancer cell lines (B) together with 0.1μg of CMV-luc reporter plasmid. After 36 hours cells were lysed and luciferase activities were measured. The relative activities were calculated by normalizing to the luc activities obtained from transfectants without CMV-Flag-AIM2. The data represent means of three independent experiments; *bars*, SD

To analyze the effect of AIM2 on biological activities, we established an inducible expression system such as a tetracycline-regulated expression system (Tet-off). Two clones were selected and shown the expression of AIM2 under an inducible condition (Fig. 2). Cell growth was monitored and shown the induction of AIM2 expression significantly slower growth rate than that of without AIM2 induction, supporting the notion that the AIM2 inhibits cell growth.

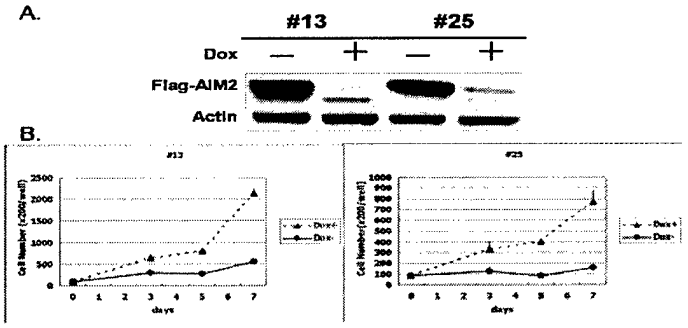
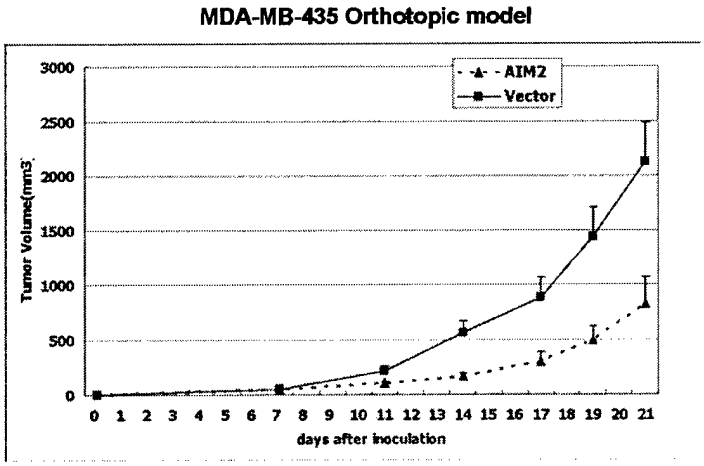


Fig. 2. The AIM2 suppressed breast cancer cells growth under inducible condition. Two stable clones, #13 and #25, of MCF-7 under tetracycline regulation system (Tet-off) were selected. AIM2 expression levels were detected in panel (A). Cells were kept in medium containing with doxycycline (1mg/ml). After seven days remove of doxycycline in medium, cells were lysed and Western block with anti-Flag antibody to detect AIM2 expression level. (B) The growth rate was measured by cell number versus time of growth. $2x10^4$ cells were plated under either with or without doxycycline, cells were collected and accounted every other days. Cell numbers were plot in panel B.

1-2-2. The AIM2 exhibited anti-tumor effect on orthotopic breast tumor model *in vivo*. To further explore the AIM2 anti-tumor activity, an orthotopic mouse tumor model was used. A human breast cancer cell line MDA-MB-435 was used to inoculate under the mammary fat pad (mfp). A recently developed non-viral gene delivery system (SN) was undertaken (17, and Appendix 5). This lipid base gene delivery, SN was established in our lab and has been shown that it can efficiently deliver the gene to orthotopic breast and prostate tumors in mice via i.v. injection (Appendix 2 and 5). Fig. 3 shows that average tumor sizes from 10 mice treated with AIM2 were significantly reduced compared with those of the group of mice treated with control vector. It suggests that AIM2 expression reduces the tumorigenicity of breast cancer cells, and systemic delivery of the AIM2 gene is a promising approach for further development as a potential therapeutic agent of metastatic cancer such as prostate cancer.

Fig. 3. AIM2 exhibits an anti-tumor effect on human breast cancer cell in an orthotopic mouse model. Six week-old female nude mice (ten mice/group) were inoculated with $2x10^6$ MDA-MB-435 breast cancer cells under mammary fat pad. Intra-tumoral gene therapy with AIM2/Liposome (20ug/8ul, every other day) started after tumors being established. Tumor sizes were measured with a caliper twice a week. The tumor volume was calculated using the formula: Volume SxSx*L*/2 where *S* is the short length of the tumor in cm and *L* is the long length of the tumor in mm ($p<0.01$).



Taken together, these results demonstrate that AIM2 possesses the anti-proliferation and anti-tumorigenic function similar to its structurally related mouse protein, p202. **In the current proposal, we will use a similar approach to further characterize the anti-tumor activity of AIM2 in prostate cancer cells (Task 1).**

1-2-3. AIM2-mediated inactivation of NF-κB may contribute to sensitize cancer cells to apoptosis induced by TNF-α. Based on our previously observation that p202-expressing cells were prone to apoptosis in response to TNF-α treatment through inhibition of NF-κB activity (Appendix 3), we hypothesize that AIM2 also inhibits NF-κB activity and therefore, can sensitize TNF-α-induced apoptosis. To test this hypothesis, we examined whether AIM2 expression could affect the NF-κB-mediated transcription activation in response to TNF-α treatment. We cotransfected CMV-Flag-AIM2 and a NF-κB-responsive promoter-reporter construct (IκB-luc) into two different breast cancer cell lines, MDA-MB-435 and MDA-MB-453 in the presence of TNF-α (Fig. 4A). As expected, IκB-luc was activated in the presence of TNF-α. Interestingly, this TNF-α-induced transcription activation was repressed by AIM2 in a dose-dependent manner. To test whether AIM2 acted on the NF-κB molecule to elicit such transcription repression, we cotransfected CMV-Flag-AIM2 with a Rel-A (a p65 subunit of NF-κB) cDNA expression vector and IκB-luc. As shown in Fig. 4B, it could greatly repress NF-κB (Rel-A)-activated IkB promoter activity. These results suggest that the transcriptional repression of TNF-α-mediated gene expression by AIM2 may be attributable to the inactivation of NF-κB by AIM2. Taken together, our preliminary results suggest that AIM2 expression may sensitize breast cancer cells to apoptosis in response to TNF-α treatment. These results also suggest a potential novel therapeutic approach that may combine TNF-α treatment in AIM2 gene therapy. **In the current proposal, we will examine whether AIM2 mediates the sensitization to TNF-α-induced apoptosis in prostate cancer cells (Task1).**

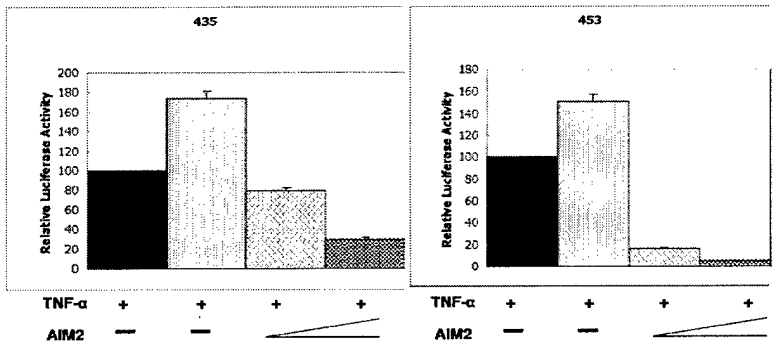


Fig. 4A. **AIM2 expression represses NF-κB-mediated transcription activation in response to TNF-α.** κB-Luciferase reporter gene (0.2 μg) and CMV-Flag-AIM2 (0, 0.5, or1.5 μg) were cotransfected into MDA-MB-453 and MDA-MB-435 cells. Thirty-six hours after transfection, cells were either left untreated or stimulated with TNF-α (20ng/ml) for 6 hours. The relative luciferase activity in IκB-Luciferase expression was calculated by setting κB-Luciferase expression in the absence of TNF-α and AIM2 as 100%.

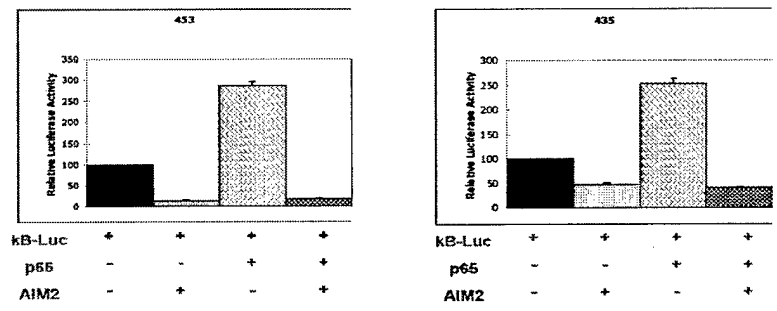


Fig. 4B. AIM2 expression represses p65-activated transcription. MDA-MB-453 and MDA-MB-435 cells were cotransfected with κB-luc and NF-κB (p65) expression vectors. The inhibitory activity of AIM2 on the induction of IκB promoter activity by p65 was assessed by cotransfection with AIM2 expression vector. Luciferase activity was measured at 48 hours after transfection. The data represent an average of two independent experiments after normalization.

1-3. Develop prostate cancer-specific expression vectors for gene therapy. In order to develop an effective gene therapy for prostate cancer, it is important that a therapeutic gene such as AIM 2 can be delivered through the route via *i.v.* injection and expresses preferentially in prostate cancer cells but not in normal cells (to prevent from potential adversary effects). As mentioned earlier, we have developed an effective gene delivery system based on non-viral liposome, SN (17, and Appendix 5), which can deliver a therapeutic gene through i.v. injection (Appendix 2 and 5). In this section, we will describe our preliminary success in constructing a broad prostate cancer-specific promoter.

To develop a prostate cancer gene therapy, the prostate cancer-specific promoters such as that of PSA (18-21), probasin (18,22 and Appendix1) and hK2 (23) have been recently developed. However, these promoters harbor certain limitations for expression in prostate cancer cells. Moreover, their activities in prostate cancer cells are, in general, lower than those of commonly used non tissue-specific virus-based promoters such as cytomegalovirus (CMV) promoter. Several modified promoters such as the modified prostate-specific probasin gene promoter, ARR_2PB have overcome this problem (22-24 and Appendix 2). As a matter of fact, the promoter activity of ARR_2PB is even higher than that of CMV promoter in response to androgen stimulation. This promoter is highly active in androgen receptor (AR)-positive prostate cancer cell line such as LNCaP; but is completely inactive in the AR-negative prostate cancer cell lines such as PC3. **Therefore, the therapeutic application of ARR_2PB promoter will be limited to the AR-positive prostate cancer only; and it cannot be applied to the AR-negative prostate cancer patients.** To overcome the limitation of ARR_2PB promoter, we intend to develop a promoter that exhibits a broad prostate cancer specificity (i.e. the activity of promoter is higher in prostate cancer cells including both AR-positive and -negative prostate cancer cells than in other normal cells or tissues). This promoter should also display a basal level activity in both AR-positive and -negative prostate cancer cells (i.e. the activity of promoter should be higher than or comparable to that of CMV promoter in prostate cancer cells). With this in mind, we made the following constructs (Figs. 5 and 6) using a human telomerase reverse transcriptase (hTERT) promoter that has been shown to possess certain specificities for cancer cells and used in cancer gene therapies including prostate cancer gene therapy (25-27). To further ensure its gene expression specifically targeted to prostate cancer cells, we took the advantage of a newly developed strategy, a two-step transcriptional amplification (TSTA) approach (28,29). The TSTA system is designed to boost the transcriptional activity of a promoter of interest (in this case, hTERTp) using a potent transcriptional activator Gal4-VP2 (Gal4 binding domain fused to the strong transcriptional activation domain of VP16), in turn, the gene of interest (in this case, the luciferase reporter gene) is amplified by G5E4T that is a Gal4 responsive elements with 5 copies of Gal4 binding sites. The several constructs in Fig. 5A were made and their promoter activities were compared in two different prostate cancer cell lines, LNCaP, AR-positive; and PC3, AR-negative cells (Fig. 5). The hTERTp activity is enhanced in both LNCaP and PC3 cell lines through the TSTA system, and is further amplified by a strong enhancer, woodchuck hepatitis virus response enhancer (WPRE) (Fig. 5 B and C) (30-33). In summary, the activity of the promoter construct (hTERTp-TSTA-WPRE) containing the TSTA system in combination

with WPRE is comparable to that of CMV construct in PC3 cells, and about 1.5-fold greater than CMV construct in LNCaP cells. Importantly, its activity remains silent in human normal lung fibroblast cells WI-38 compared with that of CMV promoter (Fig. 5D). Thus, the "chimeric" hTERTp-TSTA-WPRE construct will be able to express a gene of interest (i.e. AIM2) in the prostate cancer cells (both AR-positive and -negative) as efficiently as that of CMV promoter, but much lower in normal cells such as WI-38. The broad prostate cancer specificity in both AR-positive and -negative cells and the null activity in normal cells/tissues will be further examined in Task 2.

Many androgen-independent prostate cancers, though are refractory to androgen, still have active AR (34-37). These tumors are androgen-independent, but they appear to remain AR dependent. In this regard, a promoter that can be enhanced by androgen/AR stimulation could be very useful for prostate cancer gene therapy as it could selectively enhance the expression of the therapeutic gene in prostate cancer cells including the androgen-dependent prostate cells as well as those androgen-independent prostate cells that still contain active AR. To accomplish this goal, the ARR_2 element (androgen receptor responsive element 2) derived from the prostate cancer-specific promoter, probasin was fused to our newly constructed "chimeric" two-step systems--phTERTp-TSTA-Luc and phTERTp-TSTA-Luc-WPRE to generate the plasmids--pARR$_2$.hTERTp-TSTA-Luc and pARR$_2$.hTERTp-TSTA-Luc-WPRE (Fig. 6A). Both of LNCaP, AR-positive cells and PC-3, AR-negative cells were subjected to test transcriptional activity under stimulating condition with a metabolically non-degradable androgen analog, R1881 for two days. The activity of ARR$_2$.hTERTp-TSTA or ARR$_2$.hTERTp-TSTA-WPRE promoter was increased in an androgen-dependent manner and reached 10-fold higher activity in LNCaP cells compared to CMV. As expected, its promoter activity does not respond to R1881 and still remain a similar activity as that of CMV in PC3 cells. Our preliminary data suggest that a hTERTp-based vector with WPRE and ARR$_2$ modification has comparable activity to CMV in both AR-positive and -negative prostate cancer cells, and this activity can be further increased in response to androgen stimulation in the AR-positive prostate cancer cells. Both ARR$_2$.hTERTp-TSTA-Luc and ARR$_2$.hTERTp-TSTA-Luc-WPRE are strong gene expressors in AR-positive prostate cells such as LNCaP (about 10 fold of CMV in response to androgen stimulation) (Fig 6B upper panel) but are virtually silent in normal cells (Fig 5D). Importantly, compared with a modified prostate cancer-specific promoter such as ARR$_2$PB that is almost silent in AR-negative cells such as PC3 (Appendix 2), the ARR$_2$.hTERTp-TSTA-Luc-WPRE construct holds the advantage that it still exhibits a strong activity (comparable to CMV promoter) in PC3 cells (Fig. 6B lower panel). **The broad prostate cancer specificity and a high transcriptional activity of these vectors will be further investigated *in vitro* and *in vivo* as discussed in Tasks 2 and 3.**

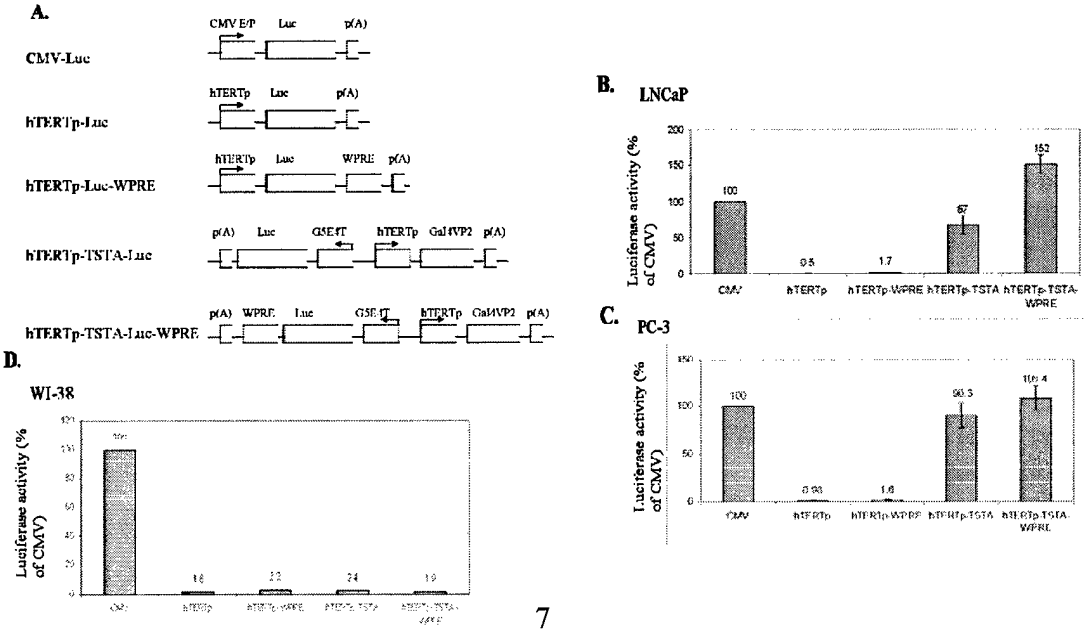


Fig. 5 Comparison of firefly luciferase activity under the control of CMV enhancer/promoter or hTERTp-based promoter composites. (A) Schematic diagram of structure of vector constructs. hTERTp: human telomerase reverse transcriptase promoter, WPRE: woodchuck hepatitis virus enhancer. Different vector constructs with similar molar quantities of pRL-TK plasmid DNA as the internal control were transiently transfected into LNCaP (B) or PC3 cells (C) or human normal lung fibroblast cells WI-38 (D). Forty-eight hours later, cells were harvested and dual luciferase activities were measured. Shown are the relative levels of activity compared to CMV construct setting at 100%. The data represent means of four independent experiments; bar, SD

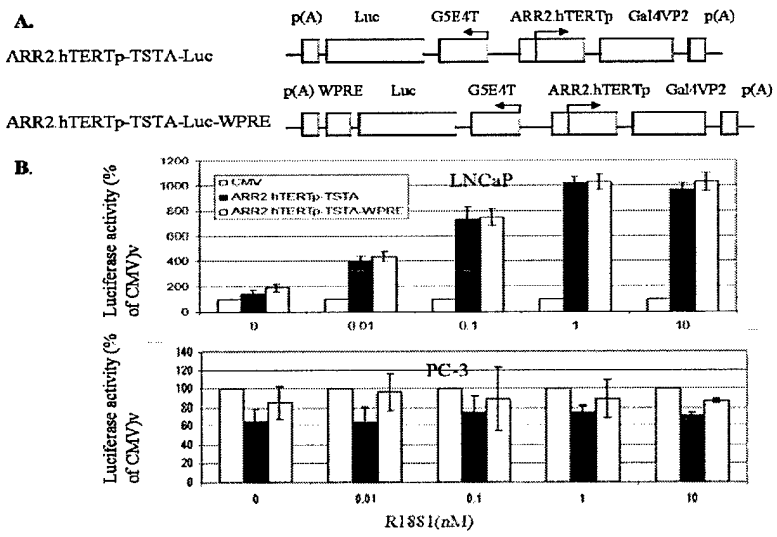


Fig. 6. The activity of androgen receptor-responsive hTERTp-based promoter. (A) Schematic diagram of structure of vector constructs. ARR_2: androgen receptor responsive element 2. (B) Different vectors together with internal control vector pRL-TK were individually transiently transfected into LNCaP (upper panel) and PC3 (lower panel) cells. Cells were treated with or without R1881, a metabolically non-degradable androgen analog, in medium containing charcoal/dextran-treated FBS at different concentrations as shown on bottom of each panel, after forty-eight hours, cells were harvested and dual luciferase activities were measured. The data represent means of four independent experiments; bar, SD.

2. HYPOTHESIS/PURPOSE

2-1. Hypothesis: The AIM2 possesses an anti-tumor activity in prostate cancer. This proposal will test the potential anti-tumor, and pro-apoptotic function of the AIM2 in prostate cancer cells using both *in vitro* and *in vivo* assays.

2-2. Purpose: To develop a novel therapeutic strategy targeting prostate cancer cells. Once we establish the anti-tumor and pro-apoptotic function of AIM2 in prostate cancer cells, we will place AIM2 gene under the control of a prostate-specific promoter to explore a possible prostate cancer-specific therapeutic effect in an orthotopic animal model. We will use our newly developed non-viral delivery system (SN liposome) that has been shown to be able to deliver gene into tumor tissue through systemically i. v. injection in an orthotopic prostate cancer animal model (Appendix 2 and 5), to deliver the prostate-specific-AIM2 construct for gene therapy.

3. OBJECTIVES

3-1. To determine the anti-tumor and the pro-apoptotic activities of the AIM2 in prostate cancer cells.

3-2. To develop a broad prostate cancer-specific expression vector for gene therapy.

3-3. To test the anti-tumor activity of AIM2 in prostate cancer cells using preclinical gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer animal model.

4. RESEARCH DESIGN AND METHODS

4-1. Task 1: To determine the anti-tumor and the pro-apoptotic activities of AIM2 in prostate cancer cells. Hypothesis: AIM2 can suppress transformation and malignancy and induce apoptosis in response to therapeutic agents, such as TNF-α, in human prostate cancer cells.

As described in our preliminary results (section 1-3), AIM2 suppresses growth of PC3 prostate cancer cells. In this Task, we would like to establish transformation suppression function of AIM2 in other prostate cancer cell lines including DU145 and LNCaP. Since PC3, DU145 and LNCaP are the three best characterized human prostate cancer cell lines, we will use all three lines in the proposed experiments throughout the entire proposal. For the sake of convenience, PC3 will be used as a representative in the following description.

4-1-1. Suppression of *in vitro* growth and transformation phenotype in prostate cancer cells by AIM2. To address this issue, we will test if the inhibitory effect of AIM2 on both anchorage-dependent and anchorage-independent growth could also be seen in prostate cancer cell lines such PC3, DU145 and LNCaP. We will transfect an AIM2 expression vector (such as CMV-AIM2) into these prostate cancer cell lines. The number of G418-resistant colonies will be scored. We expect that the number of G418-resistant colonies will be significantly lower in the AIM2 transfection. Based on our previous experience with breast cancer cell lines, we will screen a large number of G418-resistant colonies in order to obtain a few AIM2-expressing clones for subsequent biological analysis. If obtainable, we would like to isolate at least 3 independent AIM2 transfectants from each cell line to avoid any potential misleading conclusion derived from the artifact of clonal heterogeneity. The biological characterization includes cell viability will be determined by MTT assay and luciferase assay, growth rate analysis, [^{3}H] thymidine incorporation assay and soft agar colonization assays. Apoptosis will be measured by FACS analysis, or by TUNEL assay. All these technique are preformed routinely in P.I.'s laboratoty. However, it is possible that no AIM2-expressing clones could be obtained. The growth arrest and pro-apoptotic activity of AIM2 may be too strong and too toxic to survive the prostate cancer cells. In that case, an inducible expression system such as a tetracycline regulated expression system will be used to isolate the AIM2-expressing clones under the condition that AIM2 expression is not induced. Recently, we have successfully established the AIM2 inducible stable clone in breast cancer cells using Tet-Off system (Fig. 2). We will employ same technique and express AIM2 in prostate cancer cell such as PC3 and LNCaP. This way, we should be able to obtain stable transfectants that will allow us to investigate the AIM2 effects by comparing biological effects in the presence or absence of inducers. Alternatively, we can also generate an adenoviral vector expressing AIM2. With the AIM2 virus available, we can also study the biological consequence of AIM2 expression in prostate cancer cell lines. We have previously established an adenoviral vector expressing p202 gene and have shown that p202 associates with an anti-tumor activity (YD Clinical Cancer Res).

4-1-2. To determine the anti-tumor activity of AIM2 in prostate cancer mouse model. The AIM2 transfectants obtained from PC3, DU145, LNCaP cells and their respective parental control cell lines will be first subjected to s.c. tumorigenicity assay using male nude mice. Tumor volumes for AIM2 transfectants will be compared with those of their parental and vector-transfected parental cells. Based on the observed anti-growth and anti-tumor activities of AIM2 in breast cancer cell lines (Figs. 1-3), we expect these AIM2 transfectants to have a much reduced growth rate and tumorigenicity *in vivo*. In addition to s.c. tumorigenicity assay, we will also use an orthotopic prostate cancer model to compare malignancy of these AIM2 transfectants with their control parental cells. This orthotopic prostate cancer model allows us to measure tumorigenicity, prostate weight, para-aortic lymph node, and survival (Appendix 2). Using the standard procedure, the survival time of tumor-bearing mice is within 2-4 months. In addition, inducible AIM2 transfectants will be used in mouse model to further elucidate the role of anti-tumor activity by AIM2. We are experienced in the inducible system both *in vitro* and *in vivo* as shown in the preliminary results (Fig. 2).

4-1-3. Pro-apoptosis activity of AIM2 in response to therapeutic agents. Our preliminary results showed that AIM2 expression represses NF-κB-mediated transcription activation in response to TNF-α. It suggests AIM2 may sensitize cells to TNF-α induced apoptosis. We will test if the AIM2-expressing prostate cancer cells obtained from PC3, DU145, and LNCaP would be prone to apoptosis when they are treated with TNF-α. The AIM2-expressing cells will be treated with TNF-α for 48 hours, and cells will be fixed and stained with propodium iodide and subjected to FACS analysis. Percentage of apoptotic

cells in AIM2 expressed cells will be compared to that of parental cells. If AIM2 could sensitize prostate cancer cells to TNF-α-induced apoptosis, we will further examine whether AIM2 also inactivates NF-κB to result in sensitization of TNF-α-induced apoptosis as observed for AIM2 and p202 in breast cancer cells (Figs. 4, Appendix 3). The results from this study will provide a scientific basis to develop a combined TNF-α and AIM2 gene therapy strategy proposed in Task 3.

4-2. Task 2: To develop a broad prostate cancer-specific promoter. Hypothesis: The ARR_2.hTERTp-TSTA-WPRE vector is a strong broad prostate cancer-specific expressing vector.

4-2-1. A broad prostate cancer-specific promoter-driven gene expression *in vitro*. As shown in Figs 5 and 6 we have developed a strong "chimeric" broad prostate cancer-specific expressing vector, ARR_2.hTERTp-TSTA-WPRE, using combination of androgen receptor-responsive element 2 of probasin promoter conjugated to hTERT promoter and TSTA-WPRE enhancing system. The promoter activity of this chimeric construct is as strong as that of CMV promoter in AR-negative prostate cancer cells, PC3 and reach as high as 10 fold of CMV promoter activity in the AR-positive prostate cancer cells, LNCaP. Importantly, its promoter activity is almost negligible compared with CMV promoter in the human normal fibroblast cells, WI-38. To ensure this promoter is broad prostate cancer-specific, we will compare its promoter activity between prostate cancer cell lines including PC3, DU145 and LNCaP and normal human cells including prostate epithelial cells PrEC, prostate stromal cells, PrSC and prostate smooth muscle cells, PrSMC. These primary cultured cells currently are available from Combrex Bio Science Baltimore, Inc. We expect the promoter activity measured by luciferase activity will be "high" in prostate cancer cells but "low" in normal cells.

4-2-2. Prostate-specific promoter-driven gene expression *in vivo*. We will use both ARR2PB-luc and ARR2hTERTp-TSTA-luc-WPRE to perform an *in vivo* experiment in which DNA vector/liposome (SN) will be i.v. injected into the animals harboring orthotopic prostate tumors. P.I. lab has been adopted this technique (Appendix 2 and 5). The luciferase activity in the animal tissue and tumors will be indicative of targeting specificity and gene expression efficiency. To investigate the prostate cancer-specific expression in vivo, human prostate cancer cell lines such as LNCaP and PC3 will be inoculated in the orthotopic site of male nude mice as described in Appendix 2. Briefly, athymic male nude mice (nu/nu) will be opened through a single mid-ventral incision under sedation and the prostate gland will be exposed. An aliquot of 30μl of PBS containing $2x10^6$ LNCaP or PC3 cells will be inoculated into the gland with a sterile syringe and 25 G needle. Such an inoculation will result in a small swelling at the site. The abdominal incision will be closed with sterile stainless steel clips. A group of four animals will be returned to a cage following recovery from the sedation and recruited for the experiment. Two weeks after inoculation, the tumor-bearing mice will be given tail vein injections of the luciferase construct complexing with SN liposome. Twenty-four hours after injection, mice will be sacrificed; protein extracts will be isolated from the tumors and different organs including liver, spleen, lung, kidney and heart. The relative luciferase activity will then be determined using a luminometer as described previously (Appendix 5). We expect that the ARR_2.hTERTp-driven TSTA construct will produce a high luciferase activity in both LNCaP and PC3-induced tumors but much lower activities in other normal organs. Since CMV has no specificity in expression pattern, we anticipate it will produce high activities in both tumors and normal tissues as shown previously (Appendix 5). By comparing the ration of tumor versus normal tissue, we expect that the ARR_2.hTERTp-driven TSTA construct will have much higher tumor specificity than that of CMV promoter. In addition, in the case of LNCaP-induced tumors, we also expect a higher luciferase activity in the tumors for the ARR_2.hTERTp construct than the CMV promoter due to its AR-positive nature. In the case of ARR_2PB-luc construct, we expect that it will behave similar to the ARR_2.hTERTp-driven TSTA construct in LNCaP-induced tumors. However, it will be silent in the PC3-induced tumors (Appendix 2).

4-3. Task 3: To test the anti-tumor activity of AIM2 in prostate cancer cells using preclinical gene therapy strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer model. Hypothesis: The AIM2 gene can be used as an anti-tumor agent in a prostate-specific gene therapy setting; and the TNF-α enhances the efficacy of AIM2 gene therapy in a combined treatment.

4-3-1. Anti-tumor activity of AIM2 gene for tumors induced by s.c. injection. The tumor-bearing mice will be established by s.c. inoculation of three cancer cell lines (PC3, DU145 and LNCaP) in male nude mice. The AIM2 expression vector driven by CMV promoter will be complexed with SN liposome. We have previously used SN liposome complexed with other tumor suppressor genes and achieved a significant therapeutic efficacy using this type of gene therapy settings (Appendix 2 and 5). Briefly, a dose of 25μg of DNA plasmid entrapped in a lipid formulation (SN) at ratio of 1:1.5 is incubated at room temperature for 30 min. The DNA/liposome complex will be delivered through intratumor injections. The mice will be treated twice a week for a period of time. The tumor size will be monitored once per week (or more, depending on situation). We expect to observe a reduction of tumor volume in the treated groups compared with the control groups. This study will provide a proof of concept whether AIM2 expression *in vivo* will suppress the growth of prostate cancer.

4-3-2. Anti-tumor activities of AIM2 gene for orthotopic prostate tumors. A more rigorous test for the effect of AIM2 in a gene therapy setting is to perform gene therapy through i.v. injection in an orthotopic prostate tumor model (Appendix 2). To test this, we will inoculate human prostate cancer cell lines such as PC3, LNCap and DU145 in the prostate of male nude mice as described in Appendix 2. We will use this model for i.v. injection therapy. One week after tumor cells are inoculated, the tumor-bearing mice will be treated with AIM2-liposome complex through tail vein i.v. injection. The potential therapeutic efficacy achieved from single injection and multiple injections (per week) will be compared. Since the mice are expected to die within 2-4 months without treatment in this orthotopic model, the mice will be sacrificed when moribund and the survival time, prostate weight, and any para-aortic lymph node metastasis will be recorded as described in Appendix 2. This experiment will test whether the complex of AIM2-SN liposome can be effectively delivered to an orthotopic prostate cancer site and suppresses the growth of prostate cancer.

4-3-3. The prostate cancer-specific AIM2 gene therapy. Once the above-mentioned promoter experiments confirm the high prostate-specific expression (Task 2), we will next construct AIM2-expression vectors ARR_2PB-AIM2 and ARR_2.hTERTp-TSTA-AIM2-WPRE. They will be used in a gene therapy setting in the orthotopic mouse model and the efficacy of the AIM2-mediated anti-tumor effect will be assessed. We will compare the therapeutic efficacy among CMV-AIM2, ARR_2.hTERTp-TSTA-AIM2-WPRE and ARR_2.PB-AIM2 constructs. We expect that the therapeutic efficacy in the PC3- and DU145- (AR-negative prostate cancer cells) induced tumor growth in the prostate of male mice will be comparable among these three constructs. However, in the LNCaP- (AR-positive prostate cancer cells) induced tumors, the ARR_2.hTERTp-TSTA-AIM2-WPRE construct will be much more effective than that of CMV-driven as the expression level in response to hormonal stimulation of ARR_2 can be 10 fold higher. In this study, it is also important to compare the expression of AIM2 in normal tissues versus prostate tumors. We expect that CMV-AIM2 through i.v. injection will produce AIM2 expression which will be widely distributed in various normal tissues such as heart, lung, liver, spleen and kidney. On the other hand, the expression of AIM2 driven by the ARR_2.hTERTp-TSTA-AIM2-WPRE will be predominately expressed in prostate tumors but not in normal tissues as CMV does. In the case of ARR_2.PB-AIM2 construct, we expect to see similar therapeutic efficacy and expression pattern as that of ARR_2.hTERTp-driven TSTA construct in LNCaP-induced tumors. But it will not be expressed in AR-negative cells, therefore, it does not possess any therapeutic efficacy on the PC3- or DU145-induced tumors.

4-3-4. Development of a combined AIM2 gene therapy with TNF-α treatment in prostate cancer orthotopic model. Once we established a AIM2-mediated sensitization to TNF-α-induced apoptosis in

prostate cancer cells (Task 1), we will then test the efficacy of a combined treatments with both prostate-specific AIM2 expression vector plus liposome and TNF-α. To do that, we will first test the concept in a s.c. model in which prostate tumors will be inoculated s.c. followed by AIM2/TNF-α intratumor injection. The efficacy of the AIM2/TNF-a treatment will be compared with that of AIM2 or TNF-α alone to determine if there is any synergistic (or additive, or no) effect on the AIM2-mediated anti-tumor activity. If the results show a cooperative effect between TNF-α and AIM2 (synergistic or additive) we will then perform a combined therapy in an orthotopic prostate cancer model. In that setting, both TNF-α and AIM2 will be delivered through i.v. injection. Again, we will compare the efficacy obtained from the combined therapy with that of the single agent treatment. All the animal studies on the AIM2 expression will be subjected to immunohistochemical staining, TUNEL assay for measurement of apoptosis. In addition, angiogenesis markers such as CD31, VEGF and bFGF will be examined. Since the NF-κB activity is essential for tumor angiogenesis and AIM2 inhibits NF-κB activity, it is likely that AIM2 may also inhibit tumor angiogenesis, which is similar to the mouse p202 gene exhibits this activity (Appendix 3 and 4).

5. DISCUSSION

The proposal is based on the encouraging preliminary results showing that AIM2 is associated with anti-tumor activity in breast cancer cells. Most of the proposed experiments such as transfection, growth assay, tumorigenicity assay, inducible promoter system, preclinical gene therapy experiments, etc. are established techniques in the PI's laboratory as shown in the preliminary results or P.I.'s publications (please see attached appendix). Thus, we do not expect technical difficulty in the current proposal. We expect within the three-year funding period, we should be able to develop a potential novel therapeutic strategy using AIM2 gene therapy and combination therapy with TNF-α in the preclinical setting which, if successful, should enable us to develop it into clinical trials (38-41). We realize the fact that both viral and nonviral gene delivery systems have their own strengths and weaknesses. We have successfully used the nonviral delivery system in gene therapy starting from preclinical settings to clinical trials (38-41). We feel that it would be more productive for us to use what we are good at, i.e., liposome/AIM2. Therefore, nonviral delivery gene therapy will be focused in this proposal. However, if other delivery system, such as AIM2 virus, is proven to be much better approaches, we will also explore the possibility of using them in this project.

Based on the encouraging data shown in the Figs. 5 and 6, we believe that the expression vector developed will be a potent and broad prostate cancer-specific expression construct for the prostate cancer gene therapy. This will be tested in both human primary cultures and animal models (Task 2) before applying to the gene therapy (Task 3). If the vector does not work as we anticipate, we will re-engineer to make it fit into our purpose. If there are other better prostate cancer-specific expression vectors available in the future, we will certainly set up collaboration with relevant scientists to test them in our animal models.

The other concern is that the TSTA system (Figs. 5 and 6) will express a non-human protein derived from Gal4VP2, which is a fusion protein with a Gal4 binding domain and a transcriptional activator domain of VP16. This non-human protein, if expressed in normal cells, might be immunogenic and induce unforeseen potential adversary effects. However, Gal4VP2 is driven by the prostate cancer-specific promoter, ARR2-hTERRp, it will be predominately expressed in prostate cancer cells. Thus, the potential adversary effects should be minimum. As a matter of fact, if it is immunogenic and expressed in the prostate cancer cells, it could be an advantage from a therapeutic point as it may facilitate host immune response to kill the cancer cells expressing this protein. All these safety issues should be experimentally tested in the standard toxicity study in the future before the initiation of a clinical trial.

Abbreviations

AIM2	Absent in melanoma. A member of human interferon-induceible gene family with a 200-amino acid repeat.
IFN	Interferon
p202	a member of mouse interferon-inducible gene family, structural related to AIM2.
TNF-α	transforming necrosis factor alpha
E2F-	a member of E2F transcription factor family that play important roles in cell cycle control and apoptosis
Rb	a tumor suppressor, its mutation and/or inactivation is associated with retinoblastoma
MSI	microsatellite instability
NF-κB	a transcription factor that is activated as an anti-apoptotic factor in response to TNF-α treatment
PC3, DU145	human androgen receptor-negative prostate cancer cell lines
LNCaP, LNCaP-r	human androgen receptor positive-prostate cancer cell lines; LNCaP-r is subline of LNCaP.
AIPC	androgen-independent prostate cancer
hK2	human kallikrein 2
PB	probasin
hTERT	human telomerase reverse transcriptase
CMV	cytomegalovirus
WPRE	woodchuck hepatitis virus response enhancer
TSTA	two-steps transcriptional amplification
ARR_2	androgen receptor responsive element 2
ARR_2PB	a modified PB promoter that directs a high prostate cancer-specific gene expression
AR	androgen receptor
SN	non-viral liposome delivery system
FACS	fluorescence-activated cell sorter
Luc	luciferase
G418	neomycin (or Genecin)
MTT	3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Tet	tetracycline

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BIOGRAPHICAL SKETCH

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NAME	POSITION TITLE
MIEN-CHIE HUNG	**PROFESSOR AND CHAIR**

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
NATIONAL TAIWAN UNIVERSITY, TAIWAN	B.S	1973	CHEMISTRY
NATIONAL TAIWAN UNIVERSITY, TAIWAN	M.S.	1977	BIOCHEMISTRY
BRANDEIS UNIVERSITY, MASSACHUSETTS	PH.D.	1983	BIOCHEMISTRY
WHITEHEAD INSTITUTE, MIT, MASS.	POSTDOC	1984-86	ONCOGENE

Positions and Honors:

Professional Experience:

1977-1978 Assistant Research Fellow, Institute of Biological Chemistry, Academia Sinica, R.O.C.
1978 -1983 Predoctoral Fellow, Department of Biochemistry, Brandeis University, Gillette Graduate Teaching Fellowship
1984-1986 Postdoctoral Fellow, Whitehead Institute/MIT, Postdoctoral fellowship from Cancer Research Institute
1986-1991 Assistant Professor, Department of Tumor Biology, The University of Texas M.D. Anderson Cancer Center
9/91-8/94 Associate Professor, Department of Tumor Biology, The University of Texas M.D. Anderson Cancer Center
9/94- Professor, The University of Texas M.D. Anderson Cancer Center
1/96-9/98 Deputy Chairman (*ad interim*), Dept. of Tumor Biology, U.T. M.D. Anderson Cancer Center
1/96- Director, Breast Cancer Basic Research Program, U.T. M.D. Anderson Cancer Center
1/96-4/99 Hubert L. and Olive Stringer Endowed Professorship, U.T. M.D. Anderson Cancer Center
9/98-12/99 Chief, Section of Molecular Cell Biology, Dept. of Cancer Biology, U.T.M.D. Anderson Cancer Center
5/99-2/00 Hubert L. and Olive Stringer Distinguished Professorship in Cancer Research, U.T. M. D. Anderson Cancer Center
12/99-2/00 Chair (*Ad interim*), Department of Molecular and Cellular Oncology, U.T. M D. Anderson Cancer Center
03/00- Ruth Legett Jones Chair, Department of Molecular and Cellular Oncology, U.T M. D. Anderson Cancer Center
03/00- Chair, Department of Molecular and Cellular Oncology, U.T. M. D. Anderson Cancer Center

Honors and Awards:

1989-90 John P. McGovern Outstanding Teacher Award U.T. H.S.C. -Houston (1992-93, 1998-99),
1993 Faculty Achievement Award in Education (1993) and in Basic Research (1998), UTMDACC
1996 Member, Pathology B Study Section (1996 – 2000) and Sub-committee C (2001-present), NIH.
1999 Member, Editorial Board, Clinical Cancer Research (1999–), Cancer Cell (2001-) and Cancer Research (2002-)
2002 Member of the Academia Sinica, Taiwan

Peer-reviewed publications (selected from 199 peer-reviewed publications):

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Zhou, B.P., Liao, Y., Xia, W., Spohn, B., Lee, M.H., and **Hung, M.C.** Cytoplasmic localization of $p21^{Cip1/WAF1}$ by Akt-induced phosphorylation in *HER-2/neu*-overexpressing cells. ***Nature Cell Biol.*** 3(3):245-252, 2001.**Accompanied by ***News and Views*** by El-Deiry, W.S. Akt takes center stage in cell-cycle deregulation. ***Nature Cell Biol.*** 3:E71-E73, 2001.

Zhou, B.P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and **Hung, M.C.** HER-2/neu induces ubiquitination via Akt-mediated MDM2 phosphorylation. ***Nature Cell Biol.*** 3:973-982, 2001.* *Accompanied by ***Research Round up*** by Brooksbank, C. Oncogenes: breaking and entering. ***Nature Reviews Cancer***, *October 1st*, 2001.

Wen, Y., Yan, D.H., Wang, B., Spohn, B., Shao, R., Zou, Y., Ding, Y., Xie, K., and Hung, M.-C. p202, an interferon-inducible protein, mediates multiple anti-tumor activities in human pancreatic cancer xenograft models. *Cancer Res.* 61:7142-7147, 2001.

Asada, S., Choi, Y., Yamada, M., Wang, S.-C., **Hung, M.-C.**, Qin, J. and Uesugi, M. External control of Her2 expression and cancer cell growth by targeting a Ras-linked coactivator. ***Proc. Natl Acad. Sci.*** 99:12747-12752, 2002.

Bourguignon, L., Lan, K-H., Singleton, P., Lin, S-Y., Yu, D-H. and **Hung, M-C.** Localizing the EGF receptor-Reply. ***Nature Cell Biol.*** 4:E22-23, 2002.

Deng, J., Miller, S. A., Wang, H.-Y., Xia, W., Wen, Y., Zhou, B., Lin, S.-Y., Li, Yan and **Hung, M.-C.** β-Catenin interacts with and inhibits NF-κB in human colon and breast cancers. ***Cancer Cell*** 2: 323-334,2002

Feng, Z., Hu, W., Chen, J.X., Pao, A., Li, H., Rom, W.N., **Hung, M.-C.,** Adair, G. M. and Tang, M.-S. Preferential DNA damage and poor repair determine ras gene mutational hotspot in human cancer. ***J. Natl. Cancer Inst.*** 94:1527-1536, 2002.*

* Accompanied by Editorial "Etiology of the mutational spectrum of ras gene in human carcinomas" by Kelley, M. J. and Littman, S. J. ***J. Natl. Cancer Inst.*** 94:1516-1517, 2002. * Accompanied by BBC news embargo "Carcinogen in cigarettes causes mutation linked to lung cancer" Oct 14, 2002.

Lee, W.-P., Wen, Y., Varnum, B. and **Hung, M.-C**. Akt is required for Axl-Gas6 signaling to protect cells from E1A-meidated apoptosis. ***Oncogene*** 21:329-336, 2002.

Ding, Y., Wen Y., Spohn. B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., **Hung, M.-C.** and Yan, D.-H. Pro-apoptotic and Anti-tumor Activities of Adenovirus-Mediated p202 Gene Transfer. ***Clin. Cancer Res.*** 8:3290-3297, 2002.

Asada, S., Choi, Y., Yamada, M., Wang, S.-C., **Hung, M.-C.,** Qin, J. and Uesugi, M. External control of Her2 expression and cancer cell growth by targeting a Ras-linked coactivator. ***Proc. Natl Acad. Sci.*** *USA* 99:12747-12752, 2002.

Yeh, S., Tsai, M.-Y., Xu, Q., Mu, X.-M., Lardy, H., Huang, K.-E., Lin, H., Yeh, S.-D., Altuwaijri, S., Zhou, X., Xing, L., Boyce, B. F., **Hung, M-C.,** Zhang, S. and Chang, C. Generation and characterization of androgen receptor knockout (ARKO) mice: an *in vivo* model for the study of androgen functions in selective tissues. ***Proc. Natl. Acad. Sci.*** *USA* 99: 13498-13503, 2002.

Tan, M., Jing, T., Lan, K.-H., Neal, C. L., Li, P., Lee, S., Fanf, E., Nagata, Y., Liu, J., Arlinghaus, R., **Hung, M.-C.** and Yu, D.-H. Phosphorylation on tyrosine-15 of $p34^{cdc2}$ by ErbB2 inhibits $p34^{Cdc2}$ activation and is involved in resistance to Taxol-induced apoptosis. ***Mole. Cell*** 9:993-1004, 2002.

Villaret, D., Glisson, B., Kenady, D., Hanna, E., Carey, M., Gleich, L., Yoo, G. H., Futran, N., **Hung, M.-C.,** Anklesaria, P. and Heald, A. E. A multicenter phase II study of tgDCC-E1A for the intratumoral treatment of patients with recurrent head and neck squamous cell carcinoma. *Head & Neck* 24:661-669, 2002.

Zou, Y.Y., Peng, H., Zhou, B.H., Wen, Y., Tsai, Y-M., Wang, S-C. and **Hung, M-C.** Systemic tumor suppression by the pro-apoptotic gene *bik*. ***Cancer Res.*** 62:8-12, 2002.

Ueno, N. T., Bartholomeusz, C., Xia, W., Anklesaria, P., Bruckheimer, E. M., Mebel, E., Paul, R., Li, S., Yoo, G., Huang, L. and **Hung, M.-C.** Systemic gene therapy in a human xenograft tumor models by liposomal delivery of the E1A gene. *Cancer Res* 62:6712-6716, 2002.

Liao, Y. and **Hung, M.-C.** Regulation of the activity of p38 mitogen-activated protein kinase by Akt in cancer and adenoviral protein E1A-mediated sensitization to apoptosis. ***Mol. Cell. Biol.*** 23:6836-6848, 2003.

Wen Y, Giri D, Yan DH, Spohn B, Zinner RG, Xia W, Thompson TC, Matusik RJ, and **Hung MC.** Prostate-specific antitumor activity by probasin promoter-directed p202 expression. Mol Carcinog. 37:130-137, 2003.

Lee, W. P., Tai, D.-I., Tsai, S.-L., Yeh, C.-T., Chao, Y., Lee, S.-D. and **Hung, M.-C.** Adenovirus type 5 E1A sensitizes hepatocellular carcinoma cells to gemcitabine. ***Cancer Res.*** 63:6229-6236, 2003

Li, Y. M., Wen, Y., Zhou, B. P., Kuo, H.-P., Ding, Q. and **Hung, M.-C**. Enhancement of Bik antitumor effect by Bik mutants. ***Cancer Res.*** 63: 7630-7633 2003

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel on page 1 of the Detailed Cost Estimate form for the initial budget period.

NAME	POSITION TITLE
Xianghuo He	Postdoctoral Fellow

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
East China Normal University, P.R. China	B.S.	1991-1995	Zoology
East China Normal University, P.R. China	M.S.	1995-1998	Biology
Medical Center of Fudan University, P.R. China	Ph.D.	2000-2003	Oncology
University of Texas MD Anderson Cancer Center	Postdoc	2003-present	Oncology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and representative earlier publications pertinent to this application. PAGE LIMITATIONS APPLY. DO NOT EXCEED THREE PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Professional Experience:

08/2003-present Postdoctoral fellow, Department of Molecular & Cellular Oncology, U.T. M.D. Anderson Cancer Center, Houston 77030. USA

07/1998-08/2000 Research Assistant, Department of Molecular Immunology & Cell Biology, Shanghai Cancer Institute, Shanghai 200032. P.R. China

Honors and Scholarships

1991-1995 Scholarship, Honor of Excellent undergraduate several times in East China Normal University

1993-1994 ECNU Natural Science Research Fellowship for College Student

11/1994 Excellent Thesis of "Da Xia Cup" in ECNU

09/1997 Honor of Excellent Graduate of East China Normal University

10/2002 First Class Scholarship of Fudan University

09/2001 Second Class Scholarship of Fudan University

10/2002 Triple A student of Shanghai Municipal

Patent

A patent on " Human Tumor-related Novel Gene CT120 and its Coding Product from Chromosome 17p13.3 Locus ". (Patent No. 02150730.9)

Publications

1. Xianghuo He, Yujun Di, Jinjun Li, Yihu Xie, Yuntian Tang, Fengrui Zhang, Lin Wei, Yu Zhang, Wenxin Qin, Keke Huo, Yuyang Li, Dafang Wan, Jianren Gu. Molecular cloning and characterization of CT120, a novel membrane-associated gene involved in amino acid transport and glutathione metabolism. Biochemical and Biophysical Research Communications, 2002, 297(3): 528-536

2. HE Xiang-huo, QIN Wen-xin, WANG Jun-ru, HU Jian, ZHU Hong-xin, WAN Da-fang, GU Jian-ren. Chromosome Localization and Genomic Organization of Five Novel Human Genes Related to Cell Growth Control. HEREDITAS, 2002, 24(2): 111-116.

3. HE Xianghuo, LI Jin-jun, XIEYi-hu, ZHANG Feng-rui, QU Shu-min, TANG Yun-tian, QIN Wen-xin, WAN Da-fang, GU Jian-ren. Expression of human novel gene CT120 in lung cancer and its effects on cell growth. Chinese Journal of Cancer. 2003, 22(2): 113-118.

4. Xianghuo He, Yihu Xie, Jinjun Li, Wenxin Qin, Dafang Wan, Jianren Gu. Altered gene expression profiles of NIH/3T3 cells induced by Human Novel Gene CT120 implicated in lung carcinogenesis. Cell Research (revised).

5. HE Xiang-huo, QIN Wen-xin, WAN Da-fang, GU Jian-ren. An Introduction to Cancer Genome Anatomy Project. Chemistry of Life, 2001, 21(3): 192-195. (Review, in Chinese)

6. Yihu Xie, Xianghuo He, Yuntian Tang, Jinjun Li, Zhimei Pan, Wenxin Qin, Dafang Wan, Jianren Gu. Cloning and characterization of human IC53-2, a novel CDK5 activator binding protein. Cell Research, 2003, 13(2): 83-91.

7. *Yuntian Tang, Xianghuo He, Yihu Xie, Genfu Yao, Jinjun Li, Dafang Wan, Jianren Gu. Molecular Cloning and Characterization of PP3105, a novel cell growth-related gene. Tumor, 2003, 23(3):171-174.*

8. HONG Jing-jun, HE Xiang-huo, YANG Jin-song. Gene chip and its application in the cancer genomics. Letters in Biotechnology, 2002, 13(2): S11-S13. (Review, in Chinese)

9. WANG Jun-ru, QIN Wen-xin, HE Xiang-huo, PAN Yong, HU Jian, WAN Da-fang, GU Jian-ren. Effects of two haplotypes of HCAP1 gene on cellular gene expression levels in a human hepatocellular carcinoma cell line Hep3B. Chinese Journal of Cancer, 2002, 21(11): 1173-1181.

10. Yujun Di, Yu Zhang, Jinjun Li, Xianghuo He, Hong Lu, Dongbing Xu, Jiqiang Lin, Keke Huo, Dafang Wan, YuYang Li and Jianren Gu. Screening of the Proteins Interacting with HCC-associated Protein 1(HCAP1). European Journal of Biochemistry, (submitted)

11. Wang junru, Qin Wenxin, Ren Gongyi, Pan Yong, He Xianghuo, Hu Jian, Wan Dafang, Gu Jianren. Cloning of HCAP1 and studies on its preliminary function. Tumor, 2002, 22(4): 263-267.

12. ZHU Hongxin, LI Jinjun, QIN Wenxin, YANG Yanhua, HE Xianghuo, WAN Dafang, GU Jianren. Cloning of a novel gene, ANGPTL4 and the functional study in angiogenesis. National Medical Journal of China, 2002, 82(2): 94-99.

13. Yihu Xie, Wenxin Qin, Xianghuo He, Yuntian Tang, Wenxin Qin, Dafang Wan, Jianren Gu. V-ATPase and its role in cancer metastasis. Chinese Medical Abstract (Oncology), 2002, 16(4). (Review, in Chinese)

14. Liu Jiaying, He Xianghuo. Detection of Acanthamoeba Genomic DNA Polymorphisms using RAPD Molecular Markers. Acta Parasitol. Med. Entomol.Sin.. 1998, 5(4): 198-204.

15. He Xianghuo, Liu Jiaying. RAPD molecular markers and its application in parasitology. Chinese Journal of Verterinary Parasitology, 1999, 7(1): 56-59. (Review, in Chinese)

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel on page 1 of the Detailed Cost Estimate form for the initial budget period.

NAME	POSITION TITLE
Xiaoming Xie, M.D., Ph.D.	Research Scientist

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Hunan Medical University, China	M.D.	1986	Medicine
Hunan Medical University, China	Ph.D.	1994	Molecular Virology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and representative earlier publications pertinent to this application. PAGE LIMITATIONS APPLY. DO NOT EXCEED THREE PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Academic Appointments:

1986-1990 Assistant and resident
Microbiology and Infectious Diseases
The Second Affiliated Hospital
Hunan Medical University, China

1994-1995 Lecturer and attending doctor
Department of Infectious Diseases
The Second Affiliated Hospital
Hunan Medical University, China

1995-1997 Visiting scholar
Gene Therapy and Liver Disease
University of Navarra, Spain

1997-1999 Postdoctoral research associate
Medicine, Pathology and Immunology
University of Florida, USA

1999-2001 Postdoctoral research associate
Immunology
Baylor College of Medicine, USA

2001-2003 Research Associate
Immunology
Baylor College of Medicine, USA

2003-present Research Scientist
Department of Molecular Oncology
The University of Texas M. D. Anderson Cancer Center
Houston, TX

Honors and Awards:

1. The first prize in the category of basic research for the 10th Annual Oncology Research Seminar. 2001
2. The 6th Conference on retroviruses and Opportunistic Infections, travel grant. USA, 1999
3. The best oral presentation award. Terapia genica del hepatocarcinoma mediate la transferencia del gen la interlukina 12 y timidin kinasa mediada por adenovirus.Madrid, 1998
4. Dr. Sir Q.W. Lee fellowship, 1997
5. Grant of Li Zhengbie, China, 1994
6. Challenge Cup of China's invention, 1993
7. Excellent article award, Hunan Science Association, China, 1992
8. Excellent Ph.D. China, 1992
9. Excellent MD. China, 1984

Publications:

1. Zheng Y, **Xie X,** et al. Reassessment of the Widal's test in patients with viral hepatitis. Hunan J Med 1990; 7(3): 167
2. Zheng X, **Xie X,** et al. Two cases of acute fulminant hepatitis with acute hemolytic anemia and leukemia reaction. Chinese J Infect Dis 1991; 9(1): 32
3. Su X, **Xie X**. A clinical study of hepatic stimulating substance (HSS) treatment on 300 patients with viral hepatitis. Chinese J Clin Hepatol 1992;8(2): 126
4. Zhang Y, X Xie, et al. T lymphocyte colony formation in patients with hepatitis A. Gongxi J Med 1992; 6(6): 4
5. **Xie X**, X Su, et al. Detection of antibodies to hepatitis C virus in patients with hepatocellular carcinoma. South China Conference on Infectious Diseases. Aug. 1992. P26
6. **v**, X Su, et al. A preliminary study of hepatitis B virus and hepatitis C virus infection in patients with hepatocellular carcinoma. Annual Meeting of Youths Proceedings 1992;p433
7. **Xie X**, X Su, et al. A comparative study of on the relationship of HBV, HCV and HDV with hepatocellular carcinoma. International Conference on Viral Hepatitis. May 6-8,1993. Beijing. Proc of Symposium 1993;p116
8. **Xie X**, Y Zheng, et al. A dynamic investigation of lymphocyte subsets in peripheral blood of the patients with hepatitis A. Hunan J Med 1993; 11(3): 83
9. **Xie X**, X Su, et al. Coinfections by HAV, HBV, HCV and HDV in-patients with chronic liver diseases and blood donors. Chinese J Clin Hepatol. 1993; 9(1): 10
10. **Xie X**, X Su, et al. Relationship between hepatocellular carcinoma and HCV or HDV. Chinese J Integ Traditional and West Med on Liver Dis 1993; 3(2):6
11. **Xie X**, X Su, et al. The association with hepatitis C virus and hepatocellular carcinoma by polymerase chain reaction. Chinese J Infect Dis 1994; 12(3): 168
12. **Xie X** X Su, et al. Detection of serological HBV markers antibody against HCV in hepatocellular carcinoma. Hunan J Med 1994; 14(2): 6
13. **Xie X**, X Su. Investigation of hepatitis C virus RNA and hepatitis B virus DNA in patients with hepatocellular carcinoma by polymerase chain reaction. Chinese J Modern Med 1994; 4: (2): 26
14. Zheng Y, **Xie X**, et al. Investigation of T lymphocyte subsets and TL-CFU in peripheral blood in patients with Hepatitis A. Chinese J Infect Dis 1994; 12(1): 24
15. **Xie X**. Doctoral dissertation: Detection of plus and minus strand of HCV RNA in sera, tumor tissues and nontumor tissues of patients with hepatocellular carcinoma by RT-nested PCR. 1994.
16. **Xie X**. Role of Hepatitis C virus in hepatocellular carcinoma. J Pathol Physiol and Clin Med. 1995;15(2);106
17. **Xie X**, X Su, and Z Zheng. Detection of plus and minus strand of HCV RNA in sera, tumor tissues and nontumor tissues of patients with hepatocellular carcinoma by RT-nested PCR. Chinese J Infect Dis 1996; 14(4)

18. Qian C, R Bilbao and **Xie X**, M Idoato and J Prieto. In vivo gene transfer to hepatocullular carcinoma in experimental animal models by adenoviral vector. J Hepatology 1996; 25(Sup 1): 137

19. **Xie X**. Patent: Innovative syringe. China Patent 1994; 15

20. **Xie X**. C Qian. Patent. Construction of recombinant adenovirus expressing IL-12.

21. **Xie X**, CE Forsmark and JYN Lau. Feasibility of adenoviral-mediated gene delivery through ERCP-the effect of bile and pancreatic juice. Hepatology 1977; 26(5): 197A

22. **Xie X**, D Turtle, J.W. Sleasman and M.M. Goodenow. HIV LAI infection in CCR5 and CXCR4 T cells of PBMC. The 6th conference on HIV and retrovirus infection.1999, p48.

23. **Xie X**, J. W. Sleasman and M.M. Goodenow. Susceptibility of Naïve and memory CD4+ T cells to human immunodeficiency virus type 1 stain LAI and AD tagged by green fluorescent protein gene. (Manuscript to J Virology)

24. Lau J Y-N, **Xie X**, MMC Lai, and PC Wu. Apoptosis and viral hepatitis. Seminar in Liver Disease 1998; 18(2):169-176.

25. Lasarte JJ, FJ Corrales, N Casares, A Lopez-Diaz de Cerio, C Qian, **Xie X**, F Borras-Cuesta, and J Prieto. Different doses of adenoviral vector expressing IL-12 enhance or depress the immune response to a coadministrered antigen: the role of nitric oxide. J Immunology, 1999; 162:5270-5277.

26. Mazzolini G, C Qian, **Xie X**, Y Sun, JJ Lasarte M, Drozdzik, J Prieto. Regression of colon cancer and induction of antitumor immunity by intratumoral injection of adenovirus expressing interleukin-12. Cancer Gene Therapy, 1999; 6(6): 514-22

27. M Drozdzik, C Qian, **Xie X**, JJ Lasarte, J Prieto. Gene therapy of hepatoma model by combination of adenovirus expressing the HSV thymidine kinase and interleukin-12. J Hepatology. 2000; 32(2): 379-86

28. Mazzolini G, C Qian, I Narvaiza, M Barajas, F Borras-Cuesta **Xie X**, M Duarte, I Melero and J Prieto. Adenoviral gene transfer of Interleukin 12 into tumors synergizes with adoptive T cell therapy both at the induction and effector level. Human Gene Therapy 2000; 11:113-25.

29. **Xie X**, CE Forsmark, and JY Lau. Effect of bile and pancreatic juice on adenovirus-mediated gene delivery: Implications on the feasibility of gene delivery through ERCP. Digestive Diseases and Science 2000; 45(2):230-36

30. **Xie X**, X Zhou, Y Liu, CYF Young, DJ Tindall, K Slawin, and D Spencer. Robust prostate-specific expression for targeted gene therapy based on human kallikrein 2 (hK2) promoter. Human Gene Therapy 2001;12(5): 549-61

31. **Xie X**, X Zhou, Y Liu, K Slawin, and D Spencer. Adenovirus-mediated tissue-targeted expression of a caspase 9-based artificial death switch for the treatment of prostate cancer. Cancer Research 2001; 61: 6795-804

32. Qian C, **Xie X**, J Prieto, et al. Gene therapy of hepatocellular carcinoma by adenovirus transfer of HSV thymidine kinase under the control of AFP promoter. Cancer Research;(in press)

33. **Xie X** and D Spencer. Prostate-specific gene therapy via adenovirus expressing E.Coli. purine nucleoside phosphorylase driven by rat probasin ARR2PB promoter Molecular Therapy (in press)

34. **Xie X** and D Spencer. Overexpression of iCaspase-9 kills LNCaP cells and its derivatives LNCaP.IR10.1 cells and LNCaP.IR10.11 cells resistant to irradiation. (in press, Mol Ther 2004)

35. Zhao, T.Rao, X. M. Xie, X.Li, L.Thompson, T. C.McMasters, K. M.Zhou, H. S. Adenovirus with insertion-mutated E1A selectively propagates in liver cancer cells and destroys tumors in vivo. Cancer Res 2003;63(12):3073-8

36. **Xie X**, Luo, ZSlawin, K. M.Spencer, D. M. The EZC-Prostate Model: Non-invasive prostate imaging in living mice. Mol Endocrinol 2003.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel on page 1 of the Detailed Cost Estimate form for the initial budget period.

NAME	POSITION TITLE
CHAO-KAI CHOU	Graduate Research Assistant

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
National Chung Hsing University	B.S.	1996	Plant Pathology
National Cheng Kung University	M.S.	1998	Microbiology & Immunology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and representative earlier publications pertinent to this application. PAGE LIMITATIONS APPLY. DO NOT EXCEED THREE PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

RESEARCH EXPERIENCE

Intern (1994)
Advisor: Doctor S. K. Green
Department of Virology,
Asian Vegetable Research and Development Center (AVRDC)

Research Assistant (1998~1999)
Supervisor: Doctor Xi-Zhang Lin
Department of Internal Medicine,
National Cheng Kung University Hospital

Visiting Scholar (1999~2000)
Supervisor: Professor Chung-Chiun Liu, Director
Electronics Design Center,
Case Western Reserve University

Research Assistant (2002~2003)
Supervisor: Professor Hsiao-Sheng Liu
Department of Microbiology and Immunology, College of Medicine,
National Cheng Kung University

Graduate Research Assistant (2003-)
Supervisor: Mien-Chie Hung, Ph.D.
Department of Molecular and Cellular Oncology
U.T. M. D. Anderson Cancer Center – Houston, TX

PUBLICATIONS

Is green fluorescent protein toxic to the living cells? H. S. Liu, M. S. Jan, C. K. Chou, P. H. Chen, N. J. Ke (1999) Biochemical and Biophysical Research Communication; 260:712-717

Saturated saline enhances the effect of electrochemical therapy. X. Z. Lin, C. M. Jen, C. K. Chou, C. S. Chou, M. J. Sung, T. C. Chou (2000) Digestive Disease and Sciences; 45(3):509-514

Electrochemical therapy – Comparison with other local treatment methods on rat model. X. Z. Lin, C. M. Jen, M. Z. Sung, J. Y. Lin, C. K. Chou, C. H. Lou (2001) Hepato-Gastroenterology ; 48:91-94

Identifying the factors and signal pathways necessary for anchorage-independent growth of Ha-*ras* oncogene-transformed NIH/3T3 T. Y. Chang, W. J. Tsai, C. K. Chou, N. H. Chow, T. H. Leu, H. S. Liu (2003) Life Science; 73(10):1265-1274

Other Support: Hung, Mien-Chie, Ph.D.

Active- Serve as a Principal Investigator or a Project Leader:

PO1 CA099031-01 04/01/2003-03/31/2007
National Cancer Institute, NIH $200,000 ADC
"Growth Factor Receptor Signaling in Breast Cancer Progression"
Program Principal Investigator, Project 1 Leader 20%
"Functionality of ErbB2/Akt Signaling in Breast Cancer"-Project 1
Director, Administrative Core 5%

The immediate goals of this PPG are to characterize the ErbB-2/PI-3K/Akt signaling pathways in the initiation and progression of breast cancer through understanding these signaling pathways in details, and their contributions to development of breast cancer. The long-term goals of this PPG are to improve strategies for the diagnosis and treatment of human breast cancer, especially in providing knowledge for better design rationale drugs to combat this deadly disease. This PPG entitled "Growth Factor Receptor Signaling in Breast Cancer Progression" has four highly integrated projects: 1) Functionality of ErbB-2/Akt signaling in breast cancer; 2) The role of the PI-3K in mammary tumorigenesis; 3) P85 as an integration point in breast cancer progression; and 4) G_2/M deregulation and anti-apoptosis by ErbB-2 and one Administrative Core. The Project 1 focuses on the role of Akt-mediated signaling and its contribution to the cell growth and tumor progression in breast cancer cells. The main goals of the Project 1 are: to examine the molecular mechanism of anti-apoptotic function of cytoplasmic $p21^{cip1/WAF1}$; to investigate the molecular mechanism of Akt-mediated p53 functions; and to study the functionality of cytoplasmic $p21^{cip1/WAF1}$ and phosphorylated MDM2.

Overlap: None

2RO1 CA058880 08/01/1997-01/31/2005 10%
National Cancer Institute, NIH $261,000 ADC
"E1A Gene Therapy in Breast Cancer"

The major goals of this grant application are: to investigate the E1A-mediated sensitization to Taxol-induced apoptosis in breast cancer cells; to investigate the E1A-mediated sensitization to TNF-α-induced apoptosis in breast cancer cells; to develop a tumor specific promoter using hTERT promoter-driven E1A for breast cancer gene therapy; and to develop E1A clinical trials for metastatic breast cancer patients.

Overlap: None

DAMD17-01-1-0071 06/01/2001-05/31/2004 10%
DOD USAMRMC $101,141 ADC

"The p202 Gene as a Tumor Suppressor in Prostate Cancer Cells"
The major goal of this grant application are: to determine the anti-tumor and the pro-apoptotic activities of p202 in prostate cancer cells; to understand the molecular mechanisms underlying the p202-mediated anti-growth, anti-tumor and potential pro-apoptotic activities in prostate cancer; and to test the anti-tumor activity of p202 in prostate cancer cells using preclinical gene therapy

strategies and to determine the efficacy of a combined treatment with TNF-α in an orthotopic prostate cancer animal model.

Overlap: None

DAMD17-02-1-0694 09/01/2002-08/31/2006 10%
Center of Excellence, DOD USAMRMC $200,000
"Development of Targeted Therapies Strategies for Breast Cancer"-Task #3 Leader (P.I.- G. N. Hortobagyi, M.D.; Co-P.I. Eva Lee, Ph.D. UC Irvine, CA)
Task 3-"Development of Bik/Bok Gene Therapies using Liposome-mediated Systemic Gene Transfer"

The major goal of this proposal is to develop targeted therapy strategies for breast cancer. Specifically, the proposal will focus on the coordinated development of four interactive Tasks: 1) to evaluate the PTEN/PI3K as a target for therapy, to identify patients appropriate for molecular therapeutics targeting the PTEN/PI3K pathway and to determine the effects of abnormalities in the PTEN/PI3K pathway on prognosis; 2) to identify molecular therapeutic targets in the DNA repair pathway regulated by BRCA1 and BRCA2; 3) to evaluate efficacy of proapoptotic Bik and Bok gene therapy using novel targeted systemic gene therapy approaches; and 4) to develop validated mouse breast cancer models for pre-clinical drug evaluation. The major goals of the task #3 are: to develop a tumor-targeted non-viral gene delivery system for breast cancer; to develop a tumor-specific gene expression system; to determine the therapeutic efficiency of tumor-specific expression of bik delivered by tumor targeted TSN delivery system; and to determine the therapeutic efficacy of the combined tumor-specific expression of bik and bok genes delivered by tumor-specific non-viral (TSN) delivery system

Overlap: None

1P50 CA83639 (PP3)-1 09/30/1999-08/31/2004 10%
National Cancer Institute, NIH $237,389 ADC
SPORE in Ovarian Cancer, UTMDACC

"E1A Gene Therapy in Ovarian Cancer"-Project #3 Leader (P.I.: R. Bast, M.D.)
The overall goal of this UT MDACC Ovarian Spore is to facilitate innovative research in the prevention, detection, and treatment of ovarian cancer leading to the elimination of this disease as a major health problem. The Spore includes five projects: 1) chemoprevention of ovarian cancer; 2) anti-angiogenesis therapy; 3) therapy with E1A; 4) therapy with anti-HER-2 monoclonal antibodies; and 5) PI3K as a target for therapy. Three Cores (Administrative, Biostatistics and Pathology) will facilitate completion of the proposed projects. The major goals of this project #3 are: to examine E1A tumor suppressor function in ovarian cancer cells; to examine the mechanism of E1A tumor suppression function in ovarian cancer cells; to test E1A-mediated chemo-sensitization in HER2/neu-oeverexpresing ovarian cancer cells; to develop E1A gene expression system specifically targeting HER2/neu-overexpressing ovarian cancer cells using HER2/neu antisense iron-responsive element; and to test E1A phase II clinical trials of HER2/neu-overexpressing ovarian cancer patients.

Overlap: None

1 P20 CA101936-01 *07/01/2003-06/30/2008* *10%*
NCI,NIH $225,000 (ADC)

SPORE in Pancreatic Cancer, MDACC
"Development of novel gene therapy for pancreatic cancer"-Project 1 Leader (P.I. -J. Abbruzzese, M.D., Ph.D.; Co-P.I.-Hung, M.-C.)
The UT MDACC SPORE in Pancrectic Cancer is composed of five research projects, three cores, a Developmental Research Program and a Career Development Program focuses on translating recent observations in molecular biology, cellular signaling, and DNA repair to patient care. The long term goal of Project 1 is to develop an effective novel therapeutic approach to treat pancreatic cancer. To achieve this goal three specific aims are proposed: 1) to continue preclinical gene therapy experiments using orthotopic animal models of pancreatic cancer to establish a therapeutically effective approach; 2) to identify pancreatic cancer-specific expressing/delivery vectors in order to selectively express the therapeutic gene in pancreatic cancer cells, thereby preferentially inhibiting pancreatic cancer cell growth; and 3) to evaluate the therapeutic efficacy of combined gene therapy and conventional chemotherapy or radiation therapy.

Overlap: None

Active- Serve as a Co-Principal Investigator or subcontractor:

10/01/2002-09/30/2003 5%
Breast Cancer Research Foundation $250, 000 (ADC)
"A Novel Apoptotic Molecule Mtd for the Treatment of Breast Cancer"--Co-P.I. (P.I.: G.N. Hortobagyi, M.D.)

The major goal of this grant application is to use hMtd gene therapy to induce breast cancer cell death in order to develop an effective gene therapy protocol to treat breast cancer. Our ultimate goal is to take this pre-clinical animal model into clinical trials to treat human breast cancer. The proposed three specific aims are: to confirm hMtd-mediated transformation suppression in breast cancer cells; to investigate the mechanisms of hMtd-induced apoptosis; and to develop a tumor specific promoter using hTERT promoter driven hMtd to examine the preclinical effect of hMtd for breast cancer gene therapy.

Overlap: None

Pending:
NIH RO1 CA109311-01 (PI: Hung), "HER2 in Breast Tumor Progression and Metastasis"
Submitted on Oct 1, 2003.

RESOURCES

FACILITIES: Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

☒ Laboratory: The Principal Investigator has a 3000 sq. ft. of laboratory space equipped with ultracentrifuges and high-speed centrifuges, fraction collectors, refrigerators and multiple freezers and chemical hoods for proposed experiments.

☐ Clinical: N/A

☒ Animal: The M. D. Anderson Cancer Center has well-equipped animal facilities located in the Bates Freeman Building that is maintained by the Department of Veterinary Medicine and Surgery. These include SPF barrier and animal housing with laminar flow for nude mice.

☒ Computer: The administrative support offices of the Department of Molecular and Cellular Oncology are equipped with appropriate compu ter hardware, software compatible for both Mac and PC systems as well as color laser printers, and are completely networked to the hospital's main computer center.

☒ Office: The Principal Investigator has a 200 sq. ft. office and is supported by the departmental secretarial pool which is comprised of an Administrative Manager, three Administrative Assistants and two secretaries who utilize five Macintosh and two Compaq computers in their offices with four high-speed laser printers.

☒ Other: Beta Counter, contrast microscope spectrophotometer (scanning, fluorescence), centrifuge (ultra, preparative, clinicals), lyophilizers, fraction collectors. Coulter counter-channelizer, dark room, warm room and cold room. A 200 sq. ft. tissue culture facility, including laminar flow hood, and water baths are available.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The M. D. Anderson Cancer Center has a fully staffed Physical Plant Department which includes machine, electronics, refrigeration and glass shops. Investigators in the Department of Cancer Biology have access to an oligonucleotide synthesizer that is located in the department, as well as to the FACS EPICSV, counter electronics and polymerase chain reaction core facility. Administrative and secretarial services are available in the department for the preparation of manuscripts and grant expenditure management.

Appendix List:

1. **Yan DH, Wen Y, Spohn B, Choubey D, Gutterman JU, Hung MC. Reduced growth rate and transformation phenotype of the prostate cancer cells by an interferon-inducible protein, p202. Oncogene. 18:807-811, 1999.**

2. **Wen Y, Giri D, Yan DH, Spohn B, Zinner RG, Xia W, Thompson TC, Matusik RJ, Hung MC. Prostate-specific antitumor activity by probasin promoter-directed p202 expression. Mol Carcinog. 37:130-137, 2003.**

3. **Wen Y., Yan, D-H., Spohn, B., Deng, J., and Hung, M.-C. Tumor suppression and sensitization to tumor necrosis factor a-induced apoptosis by an interferon inducible protein, p202 in breast cancer cells. *Cancer Res.* 60:42-46, 2000.**

4. **Wen, Y., Yan, D.H., Wang, B., Spohn, B., Shao, R., Zou, Y., Ding, Y., Xie, K., and Hung, M.-C. p202, an interferon-inducible protein, mediates multiple anti-tumor activities in human pancreatic cancer xenograft models. *Cancer Res.* 61:7142-7147, 2001.**

5. **Zou Y, Peng H, Zhou B, Wen Y, Wang SC, Tsai EM, Hung MC. *Systemic tumor suppression by the proapoptotic gene bik. Cancer Res. 62:8-12, 2002. *Correction 62:4167, 2002.**

Oncogene (1999) 18, 807–811

http://www.stockton-press.co.uk/onc

Reduced growth rate and transformation phenotype of the prostate cancer cells by an interferon-inducible protein, p202

Duen-Hwa Yan[1,2], Yong Wen[1], Bill Spohn[1], Divaker Choubey[3,4], Jordan U Gutterman[3] and Mien-Chie Hung[1,2]

Departments of [1]Tumor Biology, [2]Surgical Oncology, and [3]Molecular Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

Interferons (IFNs) can exert cytostatic and immunomodulatory effects on carcinoma cells. In particular, growth inhibition of human prostate carcinoma by IFNs has been demonstrated both *in vitro* and *in vivo*. p202 is a 52 kd nuclear phosphoprotein known to be induced by IFNs. In this report, we showed that the expression of p202 was associated with an anti-proliferative effect on human prostate cancer cells. More importantly, cells that expressed p202 showed reduced ability to grow in soft-agar, indicating a loss of transformation phenotype. Our data suggest that p202 is a growth inhibitor gene in prostate cancer cells and its expression may also suppress transformation phenotype of prostate cancer cells.

Keywords: p202; interferon-inducible protein; prostate cancer; PC-3; growth retardation; anchorage-independent growth

Introduction

In American men, prostate carcinoma is the most frequently diagnosed malignancy and is the second leading cause of cancer death after lung cancer. It has been estimated about 209 900 men in the US. will be diagnosed with prostate cancer each year; 41 800 will die of the disease (Von Eschenbach *et al.*, 1997). The use of cytokines, e.g. interferons (IFNs), as immunotherapeutic agents has been demonstrated in several types of human cancers (Carreno and Mora, 1987; Figlin *et al.*, 1993; Fraker and Alexander, 1994; Rosenberg *et al.*, 1988). Three major defined groups of IFNs are IFN-α, IFN-β and IFN-γ (Pestka *et al.*, 1987). IFN-α is a group of closely related proteins with approximately 75% homology in their amino acid sequences. They are produced upon exposure to viruses, bacteria, and double-stranded RNA and they share the same cell membrane receptor with IFN-β (Balkwill, 1989). *In vitro* studies with the hormone-refractory human prostate cancer cell lines showed a direct anti-proliferative effect by IFN-α (Fidler, *et al.*, 1987; Sica *et al.*, 1989; Sokoloff *et al.*, 1996). Although this anti-proliferative effect of IFNs on prostate cancer cells has been recognized, the mechanism of this growth inhibition remains unclear. Moreover, an anti-tumor function of IFN-α was demonstrated in the treatment of human prostate cancer-nude mice xenografts (van Moorselaar *et al.*, 1991).

Mouse p202 gene is encoded by one of the six or more structurally related IFN-α-inducible genes of the gene 200 cluster (Lengyel *et al.*, 1995; Sen and Lengyel, 1992). Other family members include p203, p204, and D3 proteins. To date, three human homologs in this family, MNDA (Dawson *et al.*, 1995), IFI16 (Trapani *et al.*, 1994), and AIM2 (De Young *et al.*, 1997) have been identified. But the human counterpart of the murine p202 has not been reported. p202 is primarily a nuclear 52 kd phosphoprotein whose expression can be induced 15–20-fold by IFN-α in cultured cells (Choubey and Lengyel, 1993). p202 has ability to bind to both double- and single-stranded DNA nonspecifically *in vitro* (Choubey and Gutterman, 1996b). Recently, the functional significance of p202 has been implicated in that p202 can interact with several important cell-cycle, signal transduction, and differentiation regulatory molecules such as retinoblastoma gene (RB) (Choubey and Lengyel, 1995), E2F-1 (Choubey *et al.*, 1996a), E2F-4, p107 and p130 (Choubey and Gutterman, 1997), fos/jun (AP-1), NFκB (Min *et al.*, 1996), a p53 binding protein (53BP-1) (Datta *et al.*, 1996), MyoD and myogenin (Datta *et al.*, 1998). These interactions may be responsible for the subsequent transcriptional inhibition of the genes whose transcriptional activation depend on these molecules (Choubey *et al.*, 1996a; Choubey and Gutterman 1997; Datta *et al.*, 1996, 1998; Min *et al.*, 1996). One of the most pronounced cellular consequences resulted by p202 expression has been the cell growth retardation as shown in the transfected cloned murine embryo cells (AKR-2B) and murine connective tissue cells (L929) (Choubey and Lengyel, 1993; Choubey *et al.*, 1996a; Min *et al.*, 1996). Furthermore, the constitutively expressed p202 inhibits G0/G1-phase progression into the S phase in NIH3T3 cells when the growth arrested cells were stimulated to proliferate (Lembo *et al.*, 1995). That observation was supported by the recent findings that the expression of another gene 200 cluster family member, p204, also inhibited cell growth in some mouse cultured fibroblasts by accumulating the p204-expressing cells at the G1/S-phase boundary of the cell cycle (Lembo *et al.*, 1998; Lengyel *et al.*, 1995). However, it is not known whether p202 may also inhibit human cancer cell

Correspondence: M-C Hung, Department of Tumor Biology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Box 79, Houston, Texas 77030, USA
[4]Current address: Department of Radiotherapy, Loyola University Chicago-Hines VAMC, 5th Avenue and Roosevelt Road (114-B), Hines, IL 60141, USA
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growth and whether p202 has the ability to suppress transformation phenotype of human cancer cells.

In light of the correlation between the therapeutic effect of IFNs on prostate cancer cells and the growth inhibitory function of the IFN-inducible gene, p202, we tested the hypothesis that an IFN-like anti-proliferative and anti-transformation effects on prostate cancer cells could be achieved by expressing p202 in these cells. In this report, we showed that p202 expression not only could decrease the growth rate but, more importantly, could reduce the ability of a human hormone refractory prostate cancer cell line, PC-3, to grow in an anchorage-independent manner, indicating a loss of transformation phenotype.

Results and discussion

The p202 expression can inhibit human prostate cancer cell growth

Since the IFN-α treatment has been shown to have an anti-proliferative effect on human hormone-refractory prostate cancer cell lines such as PC-3 and DU145 (Fidler *et al*., 1987; Sica *et al*., 1989; Sokoloff *et al*., 1996), we first tested if the expression of the IFN-α-inducible protein, p202, was sufficient to mediate a growth inhibitory effect on these two human prostate cancer cell lines. A p202 cDNA expression plasmid (CMV-p202) (Choubey *et al*., 1996a) was transfected into PC-3 and DU145. After 3 weeks of G418 selection, the drug-resistant colonies were scored by crystal violet staining. A dramatic reduction, i.e. more than 90% reduction (Table 1), in the number of G418-resistant colonies was observed in both cell lines transfected with CMV-p202 (p202) as compared to the pcDNA3 vector control (Figure 1A). It is possible that the reduction of colony numbers in p202-transfected prostate cancer cells may be caused by the p202-mediated growth retardation and/or apoptosis. To examine these possible mechanisms and to characterize the function of p202, we attempted to isolate the p202-expressing stable cell lines. For PC-3, out of twenty randomly selected p202-transfected clones, we identified four clones that express p202 protein by western blot with a polyclonal antibody against p202 (Choubey and Lengyel, 1993): a modest expressor, p202-1, and three high expressors, p202-2, -3 and -4 (Table 1 and Figure 1B). The control cell lines, PC-3, and the pooled PC-3 colonies transfected with pcDNA3 vector (pcDNA3-pooled), have no detectable endogenous level of p202 protein. As expected, the p202-pooled showed little but detectable p202 protein expression. The positive and negative controls shown in Figure 1B were lysates isolated from AKR-2B cells treated with (+) or without (−) IFN-α, respectively (Choubey and Lengyel, 1993). It has been previously shown that the p202 antibody also recognizes a 68 kd nonspecific protein which can be used as an internal control for normalizing the sample loading (Choubey and Lengyel, 1993). We have also attempted to isolated the p202-expressing DU145 clones. However, out of 20 G418-resistant colonies screened, none expressed p202 protein (Table 1). It is possible that DU145 cells may be more sensitive to the p202 expression than PC-3 cells since DU145 cells were more sensitive to the IFN-α-induced growth inhibition than PC-3 cells (Sokoloff *et al*., 1996). To examine the growth inhibitory function of p202 in prostate cancer cells, we compared the growth rates of

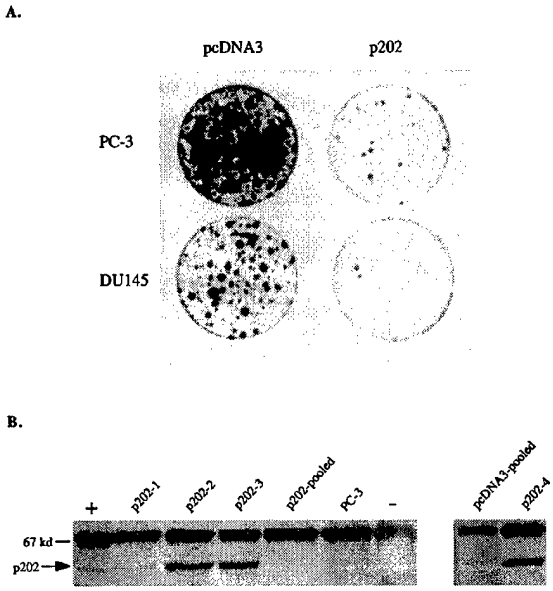

Figure 1 (A) p202 inhibits colony formation of PC-3 and DU145 cells. Both cell lines were transfected with either the vector (pcDNA3) or CMV-p202 (p202) followed by G418 selection. The G418-resistant colonies were visualized by crystal violet staining. (B) Western blot analysis of the p202-expressing PC-3 stable transfectants. The p202 protein (52 kd), as indicated by an arrow, was detected in four clones: a modest expressor, p202-1, and three high expressors, p202-2, -3 and -4. The parental, PC-3, and the vector control, i.e. a pooled G418-resistant colonies transfected with vector (pcDNA3-pooled), do not have detectable endogenous p202 protein expression. A pooled G418-resistant colonies transfected with CMV-p202 (p202-pooled) has little but detectable level of p202 protein. The p202 protein controls are the lysates isolated from AKR-2B cells treated with (+) or without (−) IFN-α. The p202 antibody could cross-react to a 68 kd nonspecific protein which migrates just above 67 kd molecular weight marker. The Western blot of pcDNA3-pooled and p202-4 was done on a different gel

Table 1 p202 expression inhibits colony formation of two human prostate cancer cell lines, PC-3 and DU145

Cell line	*% Reduction of G418-resistant colonies*[a]	*No of p202-expressing colonies/ No of G418-resistant colonies screened*[b]
PC-3	92.6±2.5	4/20
DU145	97.9±1.5	0/20

This table summarized data obtained from two independent experiments. [a]G418-resistant colonies obtained from CMV-p202 transfection as a percentage of the number of colonies obtained from pcDNA3 transfection. [b]p202-expressing colonies identified by Western blot using p202 antibody

the p202-expressing PC-3 cells with that of the control cells. Figure 2A shows that the high expressors, p202-2, -3 and -4 have a significantly slower growth rate than that of the controls, i.e. PC-3 and the pcDNA3-pooled, suggesting that the expression of p202 may be responsible for the decreased growth rate in PC-3 cells. It is interesting to note that p202-1, which has a modest level of p202 expression (Figure 1B), grew at an intermediate rate between the high expressors and the control cell lines, suggesting a p202 dose-dependent growth inhibition. Another growth assay, MTT assay (Hansen *et al.*, 1989), was also performed in these cell lines and the p202-expressing cells also showed a reduced growth characteristics than the control cell lines (data not shown). Since the growth rate is the net result of two competing processes, i.e. cell replication and cell death, it is possible that the reduced growth rates may be caused by the p202-induced anti-cell replication and/or apoptosis in these cells. To test these possibilities, we have measured the percentage of the apoptotic cells in each asynchronized cell population by using Flow Cytometry Analysis. No significant apoptosis could be observed in all these cell lines under the normal growth condition (data not shown), suggesting that apoptosis may not play a significant role in the p202-mediated growth inhibition in PC-3 cells. Since DNA synthesis rate has been used as an indicator for the rate of cell replication (Yu *et al.*, 1993), we then determined if p202 may inhibit cell growth by reducing the DNA synthesis rate in PC-3 cell. [^{3}H]thymidine incorporation assay was employed to measure the DNA synthesis rate in each cell line. Figure 2B shows that the high expressors, p202-2, -3 and -4 exhibited much slower DNA synthesis rate than that of the control cell lines. Again, p202-1 showed an intermediate rate of DNA synthesis. Taken together, the data strongly suggest that the p202-mediated growth retardation in PC-3 cells may be primarily resulted from a reduced rate of cell replication.

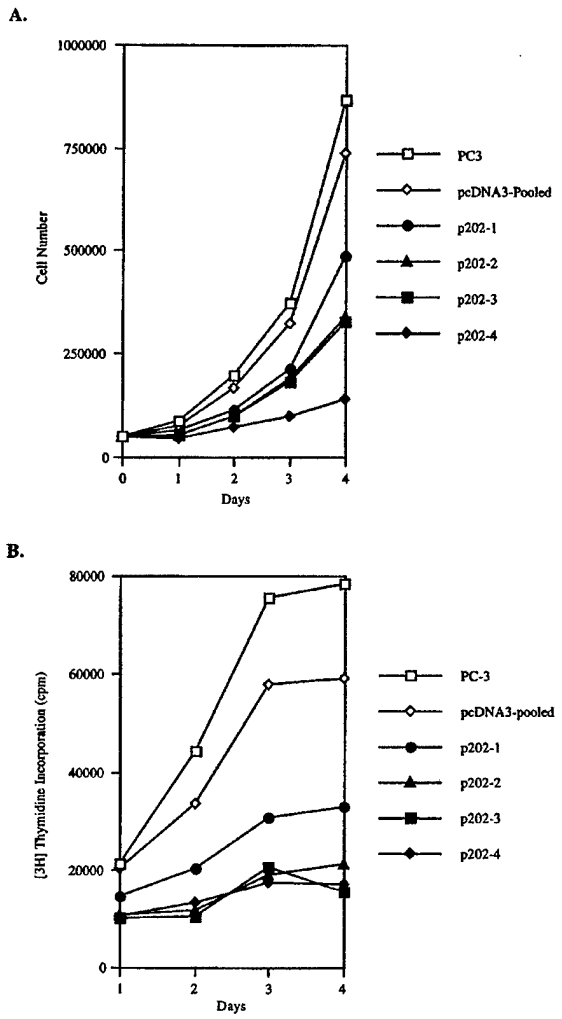

Figure 2 The reduced growth rate of the p202-expressing PC-3 cells. Four p202-expressing PC-3 stable cell lines, p202-1, -2, -3 and -4, as well as the controls, PC-3 and pcDNA3-pooled were monitored for their growth characteristics. (**A**) The growth rate was measured by cell number versus time of growth. (**B**) The DNA synthesis rate was measured by [^{3}H]thymidine incorporation versus time of growth

The p202 expression can inhibit the anchorage-independent growth of human prostate cancer cells

Since IFN-α has been shown to have an anti-tumor activity in human prostate cancer cells (van Moorselaar *et al.*, 1991), we tested if p202 expression could suppress the transformation phenotype of PC-3 cells, e.g. anchorage-independent growth. Using an *in vitro* soft-agar colonization assay (Figure 3A), we showed that while the control cell lines, PC-3 and pcDNA3-pooled, could readily form colonies in soft agar, the p202-expressors, p202-1, -2, -3 and -4, showed a greatly reduced ability to form colonies in soft agar (Figure 3B). This result suggests that the p202 expression could diminish the ability of PC-3 to grow in an anchorage-independent manner. Interestingly, the modest expressor, p202-1, like the high expressors, has lost most of its ability to form colony in soft agar, suggesting that the expression of p202 is more potent in repressing transformation than in inhibiting cell growth.

In this report, we described that the IFN-α-inducible protein, p202, not only could inhibit the growth of human hormone-refractory prostate cancer cells by reducing the DNA synthesis rate in the cells but also could abolish the ability of these cells to grow in soft agar. Thus, the p202 expression may be responsible for the loss of transformation phenotype in these prostate cancer cells. The mechanisms by which p202 inhibits cell growth are not yet well defined. However, it is conceivable that the p202-mediated inhibition of DNA synthesis (Lembo *et al.*, 1995; and this study) may be caused by the downregulation of the S-phase genes whose activation depends on the transactivation function of E2Fs. The expression of p202 abolishes E2F function because p202 interacts with the DNA binding domains of E2Fs (Choubey *et al.*, 1996a; Choubey and Gutterman, 1997), preventing E2Fs from activating the transcription of the S phase genes. How p202 inhibits transformation phenotype such as anchorage-independent growth of prostate cancer cells remains unknown. With the p202-expressing prostate cancer cell lines available, we have begun to investigate the molecular

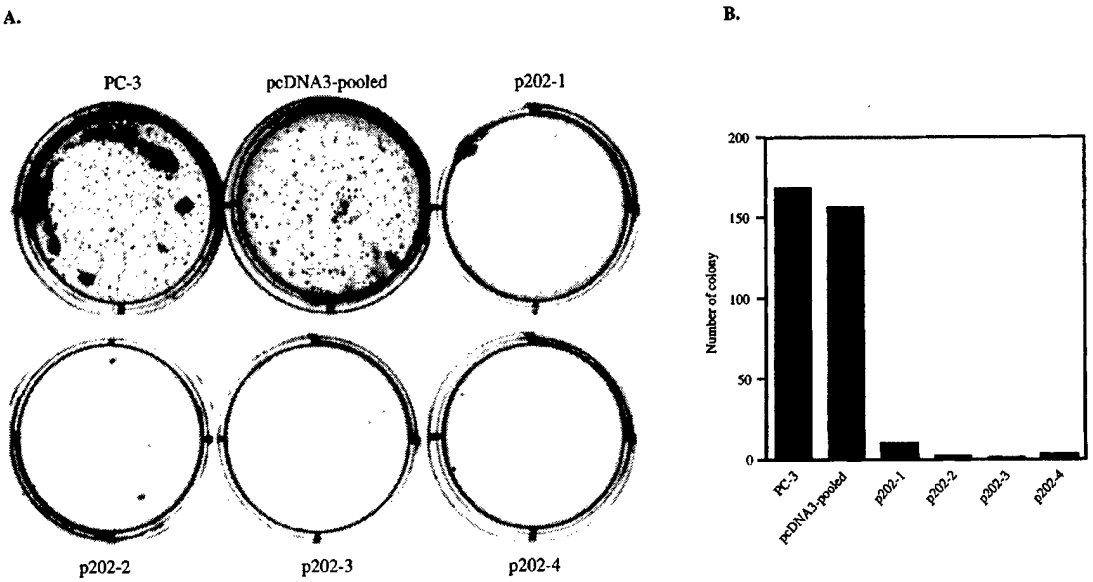

Figure 3 The p202-expressing PC-3 cells exhibit poor growth in soft-agar. (**A**) The p202-expressing PC-3 stable cell lines, p202-1, -2, -3 and -4, as well as the controls, PC-3 and pcDNA3-pooled were monitored for their ability to grow in soft-agar. After 3 weeks of incubation, the colonies were visualized by staining with p-iodonitrotetrazolium violet. (**B**) The bar diagram shows the number of soft agar colony formed by each cell line in **a**

mechanisms that underlie the anti-proliferation and the anti-tumor functions of p202.

Materials and methods

Cell lines and plasmids

Two human prostate cancer cell lines, PC-3 (Kaighn *et al.*, 1979) and DU145 (Stone *et al.*, 1978), were cultured in DMEM/F12 media supplemented with 10% fetal bovine serum. The plasmids, pcDNA3 (Invitrogen, San Diego, CA, USA) and CMV-p202 (Choubey *et al.*, 1996a), were transfected into PC-3 and DU145 cells. After 3 weeks of G418 selection (0.5 mg/ml), the drug-resistant colonies were scored by crystal violet staining (Figure 1A). Four p202-expressing PC-3 clones, i.e. p202-1, -2, -3 and -4, were identified by using Western blot with a polyclonal antibody against p202 protein (Choubey and Lengyel, 1993). The G418-resistant colonies from either pcDNA3 or CMV-p202 transfection were pooled as controls.

The growth assays

1. Direct cell counting. Cells growing in logarithmic phase in DMEM/F12 media supplemented with 10% fetal bovine serum were trypsinized, counted, and seeded in 60 mm culture dishes at 50 000 cell per dish. At 24 h intervals the cells were trypsinized and an aliquot was counted on a Coulter Counter ZM (Coulter Corporation, Miami, FL, USA). All counts were done in triplicate. 2. [^{3}H]thymidine incorporation assay. This assay was carried out as described previously (Yu *et al.*, 1993). Every data point was done in quadruplicate.

Soft-agar assay

Aliquots of cells (1×10^4) were mixed at 37°C with 0.5% agarose (Sea Plaque, low gelling temperature, FMC Bioproducts, Rockland, ME, USA) in complete media and gelled at 4°C for 15 min over a previously gelled layer of 1% agarose in complete media in six well dishes. After incubation for 3 weeks, 200 μl of 1 mg/ml p-iodonitrotetrazolium violet was added and incubated for an additional 24 h.

Acknowledgements
This work was supported by grants from NIH CA 58880, the Department of Defense 96-I-6253, and MD Anderson Cancer Center Prostate Cancer Research Program (to M-CH), NIH CA69031 (to DC), and the Clayton Foundation for Research (to JUG).

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MOLECULAR CARCINOGENESIS 37:130–137 (2003)

Prostate-Specific Antitumor Activity by Probasin Promoter-Directed p202 Expression

Yong Wen,[1] Dipak Giri,[1] Duen-Hwa Yan,[1] Bill Spohn,[1] Ralph G. Zinner,[2] Weiya Xia,[1] Timothy C. Thompson,[3] Robert J. Matusik,[4] and Mien-Chie Hung[1]*

[1]*Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas*
[2]*Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas*
[3]*Scott Department of Urology, Baylor College of Medicine, Veteran Affairs Medical Center, Houston, Texas*
[4]*Department of Urologic Surgery, Vanderbilt University, School of Medicine, Nashville, Tennessee*

p202, an interferon (IFN) inducible protein, arrests cell cycle at G_1 phase leading to cell growth retardation. We previously showed that ectopic expression of p202 in human prostate cancer cells renders growth inhibition and suppression of transformation phenotype in vitro. In this report, we showed that prostate cancer cells with stable expression of p202 were less tumorigenic than the parental cells. The antitumor activity of p202 was further demonstrated by an ex vivo treatment of prostate cancer cells with p202 expression vector that showed significant tumor suppression in mouse xenograft model. Importantly, to achieve a prostate-specific antitumor effect by p202, we employed a prostate-specific probasin (ARR_2PB) gene promoter to direct p202 expression (ARR_2PB-p202) in an androgen receptor (AR)–positive manner. The ARR_2PB-p202/liposome complex was systemically administered into mice bearing orthotopic AR-positive prostate tumors. We showed that parenteral administration of an ARR_2PB-p202/liposome preparation led to prostate-specific p202 expression and tumor suppression in orthotopic prostate cancer xenograft model. Furthermore, with DNA array technique, we showed that the expression of p202 was accompanied by downregulation of G_2/M phase cell-cycle regulators, cyclin B, and p55cdc. Together, our results suggest that p202 suppresses prostate tumor growth, and that a prostate-specific antitumor effect can be achieved by systemic administration of liposome-mediated delivery of ARR_2PB-p202. © 2003 Wiley-Liss, Inc.

Key words: p202; tumorigenecity; probasin; cyclin B; p55cdc

INTRODUCTION

The interferon (IFN) family of cytokines plays a crucial role in host defense system against viral, bacterial, and parasitic infections and certain tumors. In addition, they also possess immunomodulatory and cell growth-inhibitory activities. There are three classes of IFN: α, β, and γ [1]. The mechanism involved in tumor suppressor activity of IFNs has not been well established. However, several IFN-inducible proteins were implicated in the process of tumor suppression [2]. Consistent with that notion, a recent report based on DNA array analysis indicates that 19 of 95 differentially down-regulated genes associated with prostate tumor progression are, in fact, IFN-inducible genes [3]. The anticellular function of IFNs has been attributed to their abilities to induce G_1 phase arrest in the cell cycle [4–6]. Human prostate cancer cells are also sensitive to the antimitotic action of IFNs [7,8]. Recent studies demonstrate the inhibitory effect of IFN-α on growth [9–11] and colony formation [8] in several human prostate carcinoma cell lines.

Besides the therapeutic effects of IFNs in certain clinical settings, there are also undesirable side effects, viz. fever, chills, anorexia, and anemia, associated with high-dose IFN, which is often required to obtain a therapeutic response [12,13]. This has impeded IFN as an effective anticancer agent. In an attempt to circumvent this disadvantage and to harvest the benefit of IFN treatment, we explored the possibility of using an IFN-inducible protein, p202 [14], as a potential therapeutic agent. p202 belongs to murine 200 amino-acid protein family. Although the physiological function of p202 is not well defined, the experimental evidence gathered so far suggests its role in cell-cycle control, differentiation, and apoptosis [15,16]. In particular,

Yong Wen, Dipak Giri, and Duen-Hwa Yan contributed equally to this work.

*Correspondence to: Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd. Houston, Texas 77030.

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Abbreviations: IFN, interferon; Rb, retinoblastoma; ARR_2PB, modified prostate–specific probasin gene promoter; Luc, luciferase; PEI, polyethylenimine; PBS, phosphate buffer saline; SN, a cationic liposome formulation; AR, androgen receptor.

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ectopic p202 expression in cells results in retardation of growth that is thought to be mediated by E2F/retinoblastoma (Rb) pathway leading to G_1 arrest [17,18].

With p202 as a therapeutic agent, we have demonstrated the multiple antitumor activities in human cancer xenograft models, including breast and pancreatic cancers [19,20]. Tumor-bearing mice treated with liposome/p202 complex had suppression of tumor growth, inhibition of angiogenesis, and metastasis. In an earlier study on human prostate cancer cells, we observed that augmented expression of p202 inhibits cellular proliferation and suppresses transformation phenotype in vitro [21]. However, it has not yet been determined whether p202 expression inhibits the tumorigenicity of prostate cancer cells and whether the p202-based gene therapy is feasible in human prostate cancer xenograft model. In this report, we showed that p202 expression reduced the tumorigenicity of prostate cancer cells. With modified prostate-specific probasin gene promoter (ARR_2PB)-p202, a p202 expression vector driven by ARR_2PB promoter [22–24], we showed prostate-specific tumor suppression by ARR_2PB-p202. p202 expression was accompanied by downregulation of G_2/M phase cell-cycle regulators, cyclin B, and p55cdc [25–27].

MATERIALS AND METHODS

Cell Lines and Plasmids

LNCaP, MCF-7, PC-3, and four p202-expressing PC-3 clones, i.e., p202-1, -2, -3, and -4 [21] were cultured in Dulbecco's modified Eagle's medium/F12 media supplemented with 10% fetal bovine serum. The p202 expression vector CMV-p202 [18] is driven by CMV promoter. To construct the ARR_2PB-luciferase (Luc) vector, the ARR_2PB promoter element (468 bp), in pBlueScript II SK+ vector [22], was ligated into the KpnI/Sac I site of the PGL3-enhancer vector (Promega, Madison, WI). The ARR_2PB-p202 was generated by replacing the *Luc* gene in the ARR_2PB-Luc with the p202 coding sequence obtained from CMV-p202 vector [18] by BamHI digest. The correct orientation was confirmed by unique restriction enzyme digestion.

Subcutaneous and Ex Vivo Tumorigenicity Assays

PC-3 vector control (pcDNA3-pool), p202-1, and p202-2 cells (1×10^6 each) in 200 μL of phosphate buffer saline (PBS) were injected subcutaneously in 4- to 5-wk-old nude mice (five mice/ten tumors/cell line) on both sides of the abdomen. Tumor sizes were measured with a caliper once a week and tumor volume was calculated with the formula Vol. = S × S × L/2, where S = the short length of the tumor in cm, and L = the long length of the tumor in cm. For ex vivo experiment: PC3 cells growing in 100 mm dishes were transfected with 10 μg of CMV-p202 DNA complexed with 22.5 μg of polyethylenimine (PEI) for 45 min. PC3 cells were mock transfected with either CMV-p202 alone or PEI alone. After transfection, the cells were washed and incubated for an additional 18 h in complete media. Cells were then trypsinized, washed in PBS, counted, and 1×10^6 cells were inoculated s.c. in two sites on the flanks of male nude mice. Tumor size was measured weekly and volume calculated.

Transfection and Luc Assays

Human prostate cancer cell lines LNCaP and PC3, and a human pancreatic cancer cell line (Panc-1), were used for the reporter assay. Cells (2×10^6) were plated into a 6-well plate the day before transfection. Using SN2 liposome as a gene delivery system, cells were transfected with 0.5 μg of ARR_2-PB-Luc plasmid or 0.5 μg of CMV-Luc plasmid. pRL-TK (0.05 μg) was cotransfected as an internal control. Cells were harvested 36 h after transfection. The Luc activity was determined with the dual Luc protocol (Promega) with a luminometer.

Immunohistochemistry

The avidin-biotin peroxidase complex technique used in this study was modified from that described previously [28]. Briefly, formalin-fixed tissue sections were deparaffinized and dehydrated in ascending grades of ethanol. The sections were treated with 0.05% trypsin for 15 min, blocked in 0.3% hydrogen peroxidase in methanol for 15 min followed by treatment with 1% (v/v) normal horse serum for 30 min. The slides were incubated overnight at 4°C with anti–p202 goat polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:25 dilution. After liberal washing with PBS, the slides were incubated with biotinylated rabbit antigoat IgG at 1:200 dilution in PBS for 60 min at room temperature. The slides were subsequently incubated for 45 min at room temperature with the avidin-biotin-peroxidase complex diluted 1:100 in PBS. The product of enzymatic reaction was visualized with 0.125% aminoethylcarbazole, which gives a red colored reaction product. For counter staining, Mayer's hematoxylin was used.

Systemic Gene Therapy in Human Prostate Cancer Xenograft Model

Athymic nude mice (nu/nu) were opened through a single mid-ventral incision under sedation and the prostate gland was exposed. An aliquot of 30 μL of PBS containing 2×10^6 LNCaP cells were inoculated into the gland with a sterile syringe and 25 G needle. Such an inoculation resulted in a small swelling at the site. LNCaP cells under such conditions gave rise to tumors in 100% of animals as observed in a pilot experiment. The abdominal incision was closed with sterile stainless steel clips. A group of four animals was returned to a cage following recovery from the

sedation and recruited for the experiment. The treatment protocol was initiated 7 days after the intraprostatic inoculation of LNCaP cells, an interval sufficient to give rise to small tumors as observed in the pilot experiment. A dose of 25 μg of ARR_2PB-p202 plasmid DNA entrapped in a lipid formulation (SN) [29] at the ratio of 1:1.5 was incubated at room temperature for 30 min. The DNA/liposome complex was intravenously injected into the tail vein. The mice were treated twice a week for a period of 1½ months and then followed by treatment once a week. The Luc control group received an equivalent dose of plasmid DNA (ARR_2PB-Luc)/liposome complex. Animals were examined weekly to assess the tumor growth.

Western Blot Analysis

Protein lysate was prepared with RIPA-B cell lysis buffer containing 20 mM Na_2PO_4 (pH 7.4), 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 2 mM Na_3VO_4, 5 mM PMSF, 1% aprotinin, and 10 μg/mL of leupeptin. The antibodies specific for human Rb, cyclin B, p55cdc, and actin (Santa Cruz Biotechnology, Inc.) were used to detect these proteins by Western blot as described previously [19].

RESULTS AND DISCUSSION

p202 Suppressed Tumorigenicity of Prostate Cancer Cells

To investigate whether p202 could exhibit growth inhibitory effect on prostate tumor in vivo, two assays were performed. The first assay employed two p202 stable cell lines derived from human prostate cancer cell line, PC-3 [21]. The second was an ex vivo tumorigenicity assay with PC-3 cells transfected with p202. As shown in Figure 1A, 16 wks postimplantation, p202-1 and p202-2 clones generated smaller tumors than that of the control, the pcDNA3-pooled cell line. In fact, the p202-2 clone failed to form tumors in mice under identical experimental conditions. The difference in tumorigenesis between p202-1 and p202-2 may be attributed to an inadequate p202 protein expression in the former [21]. To rule out the possible contribution of clonal heterogeneity on the observed effects, we performed an ex vivo tumorigenicity assay in which PC-3 cells were transiently transfected with a p202 expression vector with a PEI vector delivery system. The transfected PC-3 cells were employed to generate subcutaneous xenografts in nude mice. The p202 transfected PC-3 cells, interestingly, showed no detectable tumor after 10 days (Figure 1B). On the contrary, the DNA control, i.e., CMV-p202 alone, was ineffective in containing tumor growth, indicating that the observed antitumor effect on PC-3 cells is attributable to p202 transfection. The vector controls, i.e., PEI alone, did not significantly affect tumor formation. Together, these results strongly suggested that p202 possesses an antitumor activity against prostate cancer cells. Importantly, it provides a scientific basis for developing a p202-based gene therapy strategy in an orthotopic human prostate cancer xenograft model.

ARR_2PB Promoter Directed Prostate-Specific p202 Expression and Tumor Suppression

To achieve prostate specific p202–mediated antitumor activity, we first tested whether an androgen receptor (AR)-responsive promoter could direct a Luc reporter gene expression in prostate cells. Because ARR_2PB promoter contains two copies of androgen response regions located upstream from a minimum PB promoter, it is highly responsive to androgen-dependent transcriptional activation [22]. We generated ARR_2PB-Luc and transfected it into two prostate cancer cell lines with (LNCaP) or without (PC-3)

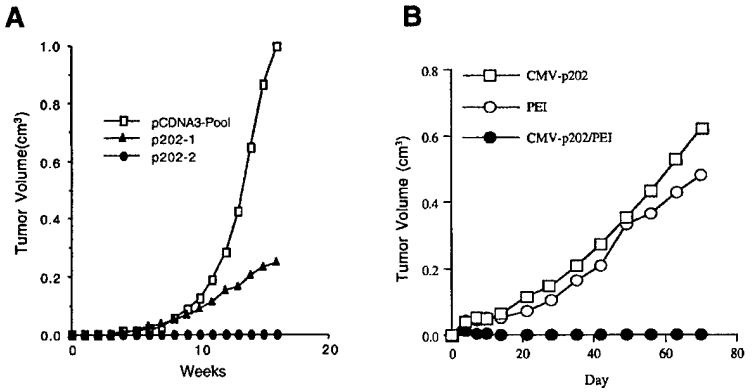

Figure 1. The antitumor function of p202 in PC-3 cells. (A) Reduced tumorigenicity of p202-expressing PC-3 cells. Nude mice (n = 5 per cell line) were injected subcutaneously with 1×10^6 cells in each of the two sides on the abdomen. Tumor size was measured each week and the tumor volume was calculated. The average tumor size at the indicated time points is presented. (B) p202 reduced the tumorigenicity of PC-3 cells ex vivo. PC-cells were transfected with CMV-p202/polyethylenimine (PEI) complex, PEI alone, or CMV-p202 alone. Eighteen hours after transfection, 1×10^6 cells were s.c. injected in both sides on the abdomen of a nude mouse.

endogenous AR expression. We used a pancreatic cancer cell line, Panc-1, as a nonprostatic control cell. As shown in Figure 2A, the relative Luc activity of ARR_2PB-Luc/CMV-Luc (a Luc gene expression vector driven by a constitutively active CMV promoter) was the highest in the AR-positive LNCaP cells, but not in AR-negative PC-3 and Panc-1 cells. This result suggested that ARR_2PB promoter activity was indeed AR-dependent [22], and thus confirmed the utility of ARR_2PB promoter to direct AR-specific gene expression in prostate cancer cells [23,24].

To test the AR-specific p202-mediated antitumor activity in orthotopic prostate cancer xenograft model, we generated a p202 expression vector driven by ARR_2PB promoter (ARR_2PB-p202). Likewise, ARR_2PB-Luc served as a negative control. The orthotopic prostate cancer xenograft model was established according to the procedure described previously [30,31]. After initiation of treatment 7 days after orthotopic tumor cell implantation in the prostate, survival time was prolonged in mice treated by ARR_2PB-p202. All mice treated with ARR_2PB-Luc were sacrificed on the 108th day post-treatment because they carried massive tumors and had reached the institutionally permissible limit for tumor burden (Figure 2B). In contrast, 100% of ARR_2PB-p202–treated mice were alive and healthy. Sixty percent (three mice) of the ARR_2PB-p202–treated mice survived on the 150th day post-treatment. To assess the antitumor activity, in an interim sacrifice protocol, three mice each from ARR_2PB-p202 and ARR_2PB-Luc treatment groups were euthanized and prostate glands dissected at day 77 of treatment. ARR_2PB-p202–treated tumors were remarkably reduced in size compared those treated by the control vector, ARR_2PB-Luc (Figure 2C). This observation explains the prolonged survival seen in mice treated by ARR_2PB-p202. The use of ARR_2PB promoter to direct expression of p202 predicts the specificity of effect. Therefore, we examined the p202 expression on tumors and organs isolated from ARR_2PB-p202–treated mice by immunohistochemical staining. The p202 protein was detected in the cytoplasm as a red colored reaction product from the enzymatic reaction with aminoethylcarbazole as the chromogen. Note abundant intracytoplasmic expression of p202 in the tumor from the mouse treated with ARR_2PB-p202 (Figure 3, left panel). The mouse treated with ARR_2PB-Luc had undetectable p202 (Figure 3, right panel). Given that p202 is primarily a nuclear protein [32], the exact reason for the predominant cytoplasmic staining of p202 is not clear. However, it is probably due to the robust expression of p202 that causes accumulation of p202 in the cytoplasm. Alternatively, because the induced p202 localizes in the cytoplasm for 30–36 h after IFN treatment before translocated into the nucleus [32], it is likely that p202 could still remain in the cytoplasm 20-h post–ARR_2PB-p202 treatment. We also examined the expression of p202 in multiple organs such as lung, liver, kidney, and heart to ascertain the nonprostatic expression, if any. There was no extraprostatic expression of p202 except the reticuloendothelial cells of lung and liver from both ARR_2PB-p202 and ARR_2PB-Luc–treated mice (data not shown and Figure 3). The p202-positive mouse reticuloendothelial cells is probably the endogenous level of p202 expression, because all 200 amino-acid protein family members are expressed in hematopoietic cells [33]. Together, the results strongly suggested that systemic delivery of ARR_2PB-directed expression vector by SN liposome could result in prostate and AR-specific antitumor activity in prostate cancer.

ARR_2PB promoter–mediated therapeutic gene expression is primarily useful for targeting AR-positive prostate cancer, which makes up a significant portion of the prostate cancer patient population. Although AR-negative prostate cancer is insensitive to androgen, in many of these cases, AR is still active [34]. It is conceivable that ARR_2PB promoter could be activated in these androgen-independent prostate tumors. In addition, ARR_2PB promoter is also responsive to glucocorticoids that have been routinely used to improve the quality of life in prostate cancer patients who failed androgen deprivation therapy [22,35]. Thus, ARR_2PB-p202 could be potentially used to achieve a prostate-specific therapeutic effect on androgen-independent prostate cancer patients who are treated with glucocorticoids.

p202 Upregulated the Hypophosphorylated Rb and Downregulated Cyclin B and p55cdc

To investigate the underlying mechanisms of the p202-mediated growth inhibition and tumor suppression in prostate cancer cells, we set out to determine (1) if Rb phosphorylation was involved in p202-mediated growth arrest because IFN treatment increases the level of hypophosphorylated (active) form of Rb [36–38]; and (2) other regulatory genes responsible for the p202-mediated growth retardation and tumor suppression that can be identified by DNA array technology. To examine the effect on Rb phosphorylation by p202, we employed Western blotting with a Rb-specific antibody to analyze the phosphorylation status of Rb in both parental and p202-expressing prostate cancer cells. Figure 4A shows that the p202-expressing cells, i.e., p202-1, -2, and -3, exhibit an elevated level of hypophosphorylated form (faster migrating band) of Rb as compared to the control, i.e., pcDNA3-pooled, in which the hyperphosphorylated form (slower migrating band) of Rb is most prevalent. Thus, one possible mechanism by which p202 induces cell growth arrest in PC-3 is by enhancing the level of hypophosphorylated Rb. Presumably, the active Rb would then inhibit E2F transactivation function by forming an Rb/E2F complex. Thus, the E2F-mediated transcription of S-phase genes might be inhibited

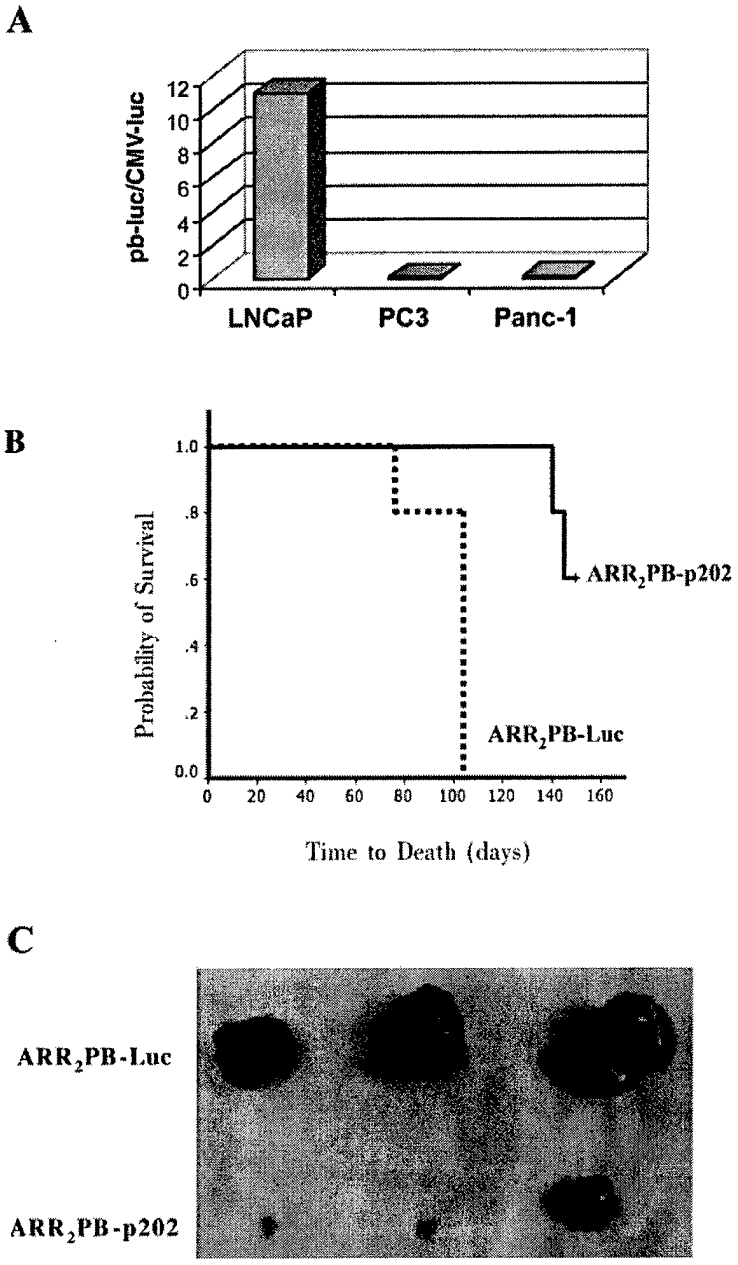

Figure 2. (A) Modified prostate-specific probasin gene promoter (ARR_2PB) activity was androgen receptor (AR)-dependent. ARR_2PB-luciferase (pb-Luc) (0.5 μg) and CMV-Luc (0.5 μg) were transfected into two prostate cancer cell lines with, for example, LNCaP, or without, for example, PC3, endogenous AR expression. A pancreatic cancer cell line, Panc-1, served as a nonprostatic cell control. The ratios of Luc activity resulted from ARR_2PB-Luc and CMV-Luc transfections were measured. pRL-TK (50 ng) was co-transfected and served as an internal control for transfection efficiency with dual Luc assay (Promega, Madison, WI). The data shown here are the average of two independent experiments. (B) Prolonged survival by ARR_2PB-p202 treatment. LNCaP orthotopic tumor-bearing mice ($n = 5$ per treatment group) were intravenously treated with ARR_2PB-p202 or ARR_2PB-Luc/SN liposome complexes. Survival rates were measured by Kaplan-Meier analysis. (C) Antitumor activity by systemic ARR_2PB-p202 treatment. Tumor suppression by ARR_2PB-p202 treatment. Representative LNCaP tumors ($n = 3$ per treatment group) are shown from mice treated with ARR_2PB-p202 or ARR_2PB-Luc/lipid formulation (SN) liposome complexes on day 77 post-treatment.

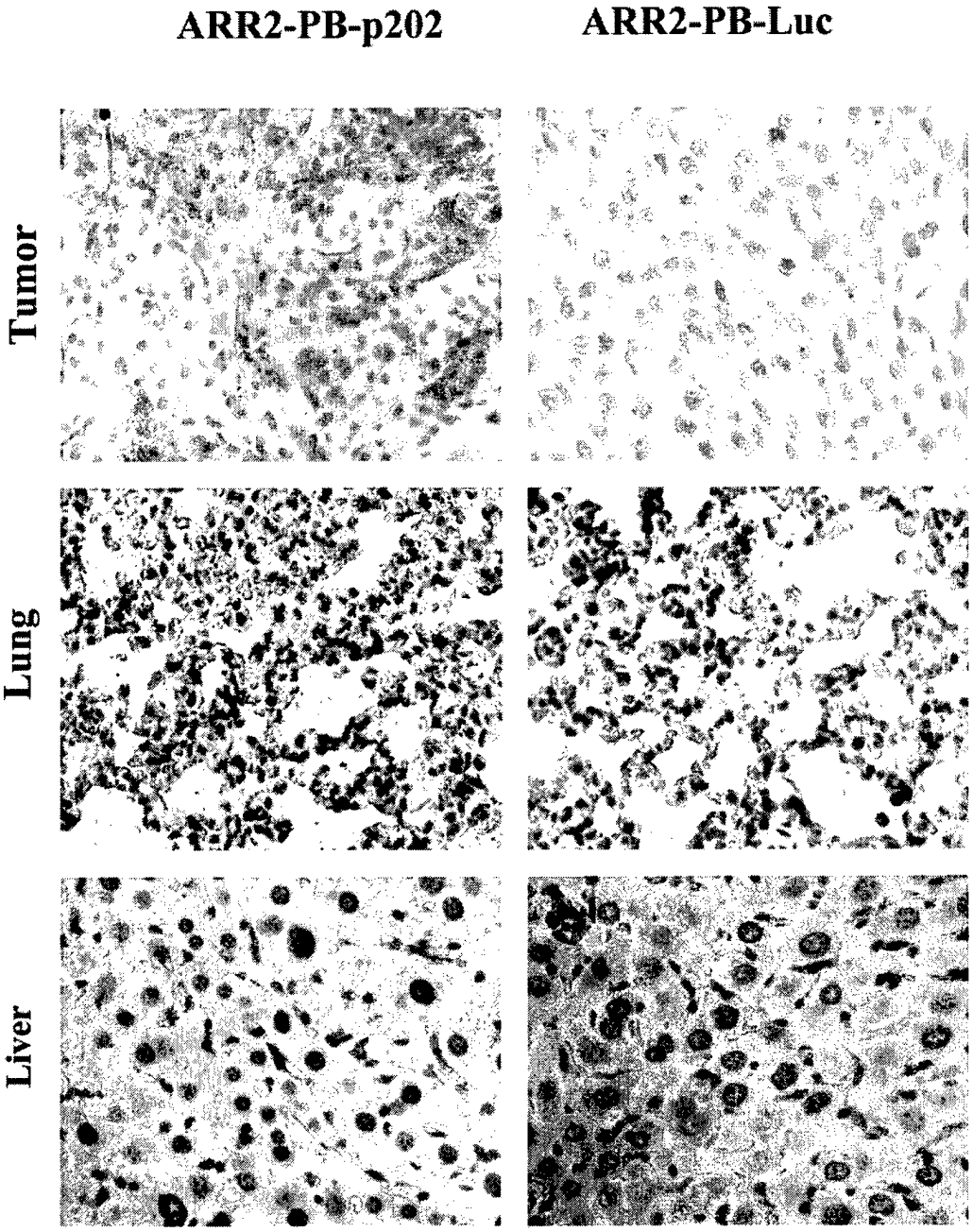

Figure 3. ARR_2PB promoter directed prostate-specific p202 expression. Formalin-fixed tumors, lung, and liver from mice 20-h post-treatment with ARR_2PB-p202 or ARR_2PB-Luc were sectioned and stained for p202 employing polyclonal anti–p202 antibodies as described in Materials and Methods.

causing G_1-phase arrest. Because p202 is a transcription modulator, it is possible that p202 could regulate certain gene expression which might be important in p202-mediated growth arrest and tumor suppression in prostate cancer cells. To identify other critical genes involved in p202-mediated antigrowth and antitumor activities, we employed DNA array technology. With RNA products obtained from PC-3 (parental control) and p202-2 (a representative p202-expressing prostate

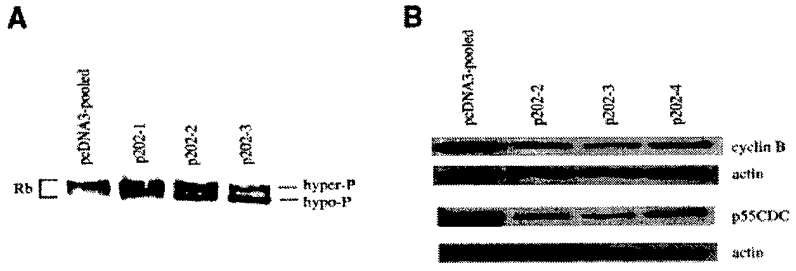

Figure 4. p202 enhanced hypophosphorylated retinoblastoma (Rb) and reduced cyclin B and p55CDC expression. Cell lysates obtained from pcDNA3-pool and p202-expressing PC-3 cell lines (p202-1, -2, -3, and -4) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently analyzed by Western blot with antibodies against Rb, cyclin B, p55cdc, and actin. The actin bands served as loading control.

cancer cell) to hybridize with DNA array filters (Clontech, Palo Alto, CA) containing 588 known genes that are involved in various cellular regulatory pathways including those of cell-cycle control, we were able to identify several candidate genes whose expression was found to be significantly influenced by p202 expression. Two such genes have been confirmed by Western blotting, i.e., cyclin B and p55cdc (Figure 4B), which showed a reduced level of expression in p202-expressing cells, as compared to the control, pcDNA3-pooled. The sample loading was similar, as indicated by the actin control. In light of the well-documented p202-mediated G_1 arrest, the reduction of cyclin B and p55cdc in p202-expressing cells is rather surprising, because cyclin B is involved in G_2/M phase transition [25] and p55cdc is required for normal metaphase-to-anaphase transition involved in late mitotic events [26,27]. It is likely that the downregulation of these two genes by p202 may contribute to the p202-mediated cell-cycle arrest. This is the first time that p202 has been implicated in involvement in G_2/M phase cell-cycle control. It is possible that the p202-associated cyclin B and p55cdc downregulation may contribute, in part, to the p202-mediated growth arrest.

In this report, we showed that p202 expression suppressed the tumorigenicity of prostate cancer cells. A subsequent ex vivo experiment with either CMV-p202/CMV-PEI complex also inhibited prostate cancer cell growth in a xenograft model. The utility of p202 as a potential therapeutic gene for prostate cancer treatment was demonstrated by the observation that prostate-specific antitumor activity can be achieved by systemically treating the prostate tumor-bearing mice with a p202 expression vector driven by a composite probasin promoter, ARR_2PB. Thus, in addition to local and systemic treatment of breast and pancreatic tumors, respectively, by using a p202 expression vector driven by a constitutively active promoter such as CMV promoter [19,20], our results suggested the feasibility of using a tissue-specific promoter to achieve p202-mediated antitumor activity in those cancer types as well. Experiments are underway to test that possibility. Given that p202 is involved in G_0/G_1 transition by targeting E2F/Rb pathway [16], it is interesting to note that G_2/M cell-cycle regulators such as cyclin B and p55cdc are downregulated by p202. Further analysis on the p202 effect on G_2/M transition by downregulation of cyclin B and p55cdc will shed light on how p202 inhibits cell proliferation.

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Advances in Brief

Tumor Suppression and Sensitization to Tumor Necrosis Factor α-induced Apoptosis by an Interferon-inducible Protein, p202, in Breast Cancer Cells[1]

Yong Wen,[2] Duen-Hwa Yan,[2] Bill Spohn, Jiong Deng, Shiaw-Yih Lin, and Mien-Chie Hung[3]

Department of Cancer Biology, Section of Molecular Cell Biology [Y. W., D-H. Y., B. S., J. D., S-Y. L., M-C. H.], Department of Surgical Oncology [D-H. Y., M-C. H.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

p202, an IFN-inducible protein, interacts with several important regulatory proteins, leading to growth arrest or differentiation. In this report, we demonstrate that, in addition to inhibiting *in vitro* cell growth, p202 can also suppress the tumorigenicity of breast cancer cells *in vivo*. Furthermore, we found that p202 expression could sensitize breast cancer cells to apoptosis induced by tumor necrosis factor α treatment. One possible mechanism contributing to this sensitization is the inactivation of nuclear factor-κB by its interaction with p202. These results provide a scientific basis for a novel therapeutic strategy that combines p202 and tumor necrosis factor α treatment against breast cancer.

Introduction

IFNs possess a wide variety of biological properties such as antivirus, antiproliferation, immunoregulation, antiangiogenesis, and antineoplasia and have been used in clinical treatment of certain cancers (1). Here, we examined the possibility of using an IFN-inducible protein, p202 (2), as a potential therapeutic substitute for IFNs. p202 is a M_r 52,000 nuclear phosphoprotein known to be a negative transcription modulator that, in most cases, inhibits transcription of its target genes by physically interacting with certain transcription activators (3–8). Like IFN treatment, constitutive expression of p202 causes G_1-S cell cycle arrest in murine fibroblast cells (9, 10). Consistent to that observation, we demonstrated previously that the enforced expression of p202 could significantly retard the *in vitro* growth of prostate cancer cells in both cell culture and soft agar (10). However, it is not known whether p202 expression could exert an antitumor effect on cancer cells. In this report, we demonstrated for the first time that p202 expression was able to inhibit tumorigenicity of human breast cancer cells *ex vivo*. Furthermore, p202 expression can sensitize breast cancer cells to apoptosis induced by TNF[4]-α and that correlates with inactivation of NF-κB by a NF-κB/p202 interaction. These results suggest a potential combined therapy using p202 and TNF-α against breast cancer.

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[2] These two authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Department of Cancer Biology, Section of Molecular Cell Biology, Box 108, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3630; Fax: (713) 794-0209.

[4] The abbreviations used are: TNF, tumor necrosis factor; NF, nuclear factor; CMV, cytomegalovirus; PEI, polyethylenimine; FACS, fluorescence-activated cell sorter.

Materials and Methods

Cell Culture, Transfection, and Colony-forming Assay. MDA-MB-453 and MCF-7 human breast cancer cell lines were obtained from the American Type Culture Collection. Cells were maintained in DMEM/F-12 (HyClone Laboratories, Inc.) supplemented with 10% (v/v) fetal bovine serum. Cells were transfected with a p202 expression vector (CMV-p202) or the control vector pcDNA3 (Invitrogen) using lipofectin (Life Technologies, Inc.) and selected in 500 μg/ml G418 (Geneticin; Life Technologies, Inc.). Western blotting using an anti-p202 polyclonal antibody (11) identified p202 stable transfectants.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay and FACS Analysis. These standard assays were done as described previously (12).

[^{3}H]Thymidine Incorporation Assay and Soft-Agar Assay. These standard assays were done as described previously (12).

Tumorigenicity Assay. Female athymic nude mice (*nu/nu*), 4–5 weeks of age, were used in this *ex vivo* experiment. Briefly, MCF-7 cells were transfected with CMV-p202 (10 μg) using PEI. Twenty-four h after transfection, cells (3×10^6) were harvested in 0.2 ml of PBS and injected into the mouse mammary fat pads. 17-β-Estradiol pellets (0.72 mg/pellet, 60-day release; Innovative Research of America, Inc.) were implanted s.c. into the mice 1-day before cell injection. The presence of estrogen is essential for MCF-7 cells to grow in mice. The size of the tumors was measured with a caliper every week, and the tumor volume was calculated using a formula: $V = 1/2 \times S^2 \times L$, where V = volume, S = the short length of the tumor, and L = the long length of the tumor in cm.

Immunoprecipitation and Immunoblotting. MDA-MB-453 (453) and 453-p202 cells were treated with 10 and 20 ng/ml of human TNF-α (R & D Systems, Inc., Minneapolis, MN) for 30 min. Cells with or without TNF-α treatment were extracted in RIPA lysis buffer without SDS on ice. Exacts were sonicated and cleared by centrifugation at 4°C. For immunoprecipitation, equivalent aliquots of cell lysates (1 mg of total protein) were incubated with 1 μg of anti-p65 antibody (Santa Cruz Biotechnology) for 4 h with gentle rotation at 4°C. Protein A-Sepharose beads (50 μl) was added for an additional 1 h. The beads were extensively washed with ice-cold RIPA buffer, and the precipitate was dissolved in a sample buffer for electrophoresis and Western blot.

Results and Discussion

To investigate a potential growth-inhibitory effect of p202 on breast cancer cells, we performed a colony-forming assay by transfecting a p202 expression plasmid driven by CMV promoter (CMV-p202) or a control vector (pcDNA3) containing neomycin-resistance gene into two human breast cancer cell lines, MDA-MB-453 (453) and MCF-7. After 3 weeks of G418 selection, the number of G418-resistant colonies was scored. A dramatic reduction in the number of G418-resistant colonies was seen in cells (MCF-7 and 453) transfected with p202 as compared with that with the control plasmid, pcDNA3 (Fig. 1*a*, *left panel*). There was at least a 75% reduction in colony number in both p202-transfected cell lines (Fig. 1*a*, *right panel*). These data suggest that p202 expression may be associated with antiproliferation

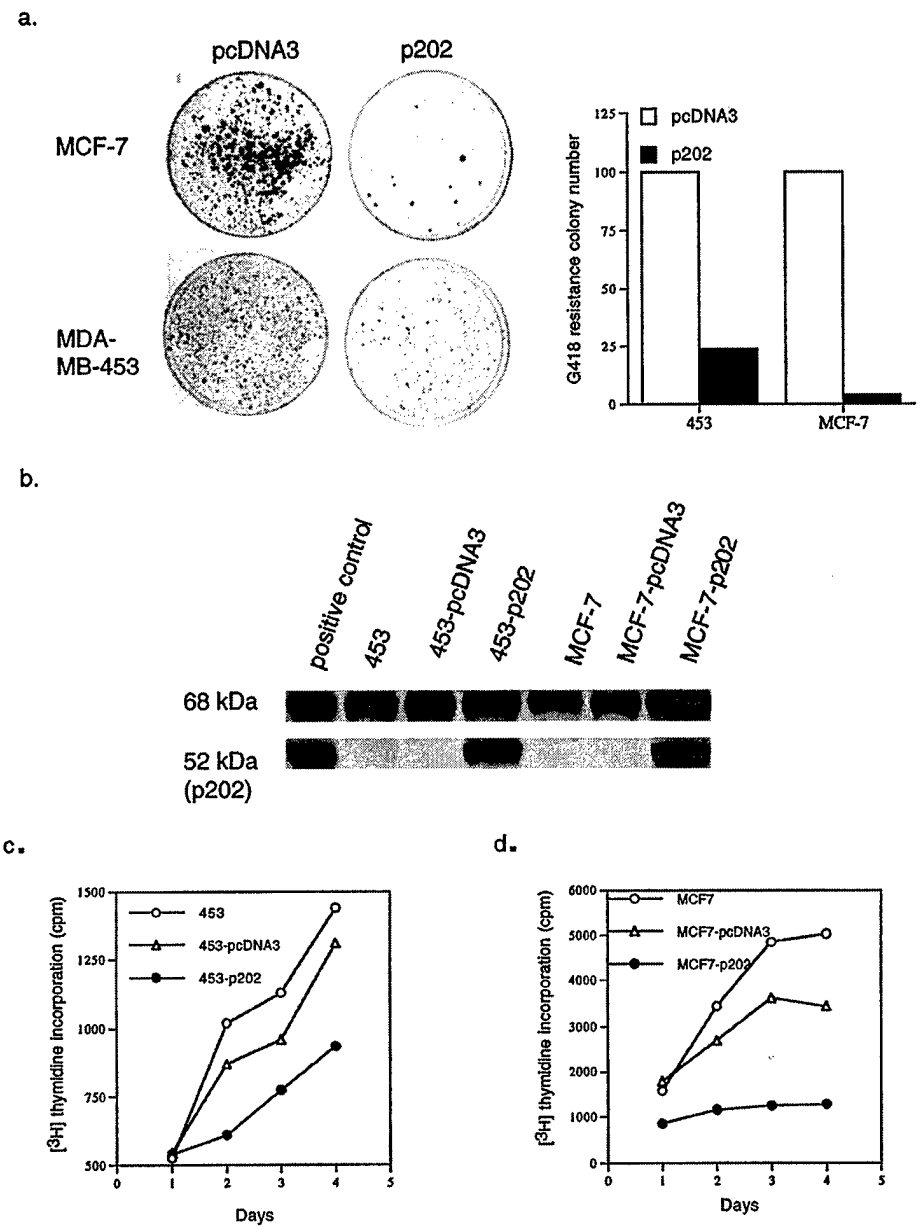

Fig. 1. Expression of p202 inhibits the proliferation of MDA-MB-453 and MCF-7 breast cancer cells. *a*, colony-forming assay. MDA-MB-453 and MCF-7 cells were transfected with either a control vector (pcDNA3) or a p202 expression vector. The colony number obtained from pcDNA3 transfection was set as 100%. *b*, Western blot analysis of the p202 stable transfectants. The M_r 52,000 protein represents p202, and the nonspecific M_r 68,000 protein cross-reacting with the antibody was used as an equal loading control. *c* and *d*, [^{3}H]thymidine incorporation assays. DNA synthesis rate was measured by the amount of [^{3}H]thymidine incorporated into the cells at each time point. The measurement was conducted in quadruplicates, and the variations within each quadruplicate are too small to be of any significance.

and/or proapoptotic activity in these breast cancer cells. To further characterize the biological effects of p202 expression on these cells, we attempted to isolate several lines of p202-expressing stable clones. Using Western blot with a p202-specific antibody (11), we were able to identify one p202-expressing stable clone (of 20) from each cell line, *i.e.*, MDA-MB-453-p202 (453-p202) and MCF-7-p202 (Fig. 1*b*). The low frequency of p202-expressing clones obtained from the G418-resistant colony supports the idea that p202 expression may cause an antiproliferation and/or proapoptotic effect on these cells. To assess these two p202-mediated biological effects, we first measured and compared the mitogenic activity between the p202 stable lines and the control cell lines using [^{3}H]thymidine incorporation assay. The p202-expressing cells (453-p202 and MCF-7-p202) exhibited a reduced DNA synthesis rate as compared with their respective control cell lines, *i.e.*, 453 and 453-pcDNA3; MCF-7 and MCF-7-pcDNA3 (Fig. 1, *c* and *d*). Similarly, the p202-expressing cells also showed a slower growth rate than the control cells as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). Thus, our data strongly suggest that p202 functions as a growth inhibitor in breast cancer cells.

To test whether p202 expression in breast cancer cells may also suppress their *in vitro* transformation phenotype defined by the ability of these cells to grow in soft agar, we then measured the number of colonies formed in soft agar by the p202-expressing cells and the control cells. As shown in Fig. 2, *a* and *b*, both 453-p202 and MCF-7-p202 exhibited >60% reduction (after 3 weeks of incubation) in colony number than those of the parental and pcDNA3 transfectant. The difference in number was not attributable to the slower growth rate of the p202-expressing cells than that of the control cells (Fig. 1, *c* and *d*), because a prolonged (6 weeks) incubation of the same plates did not yield more colonies. Rather, it represents a real loss of anchorage-independent growth, *i.e.*, an *in vitro* transformation phenotype, of these p202-expressing cells.

One of the most critical biological properties determining the potential application of a tumor suppressor gene in cancer therapy is its ability to reduce tumorigenicity *in vivo*. To test a possible antitu-

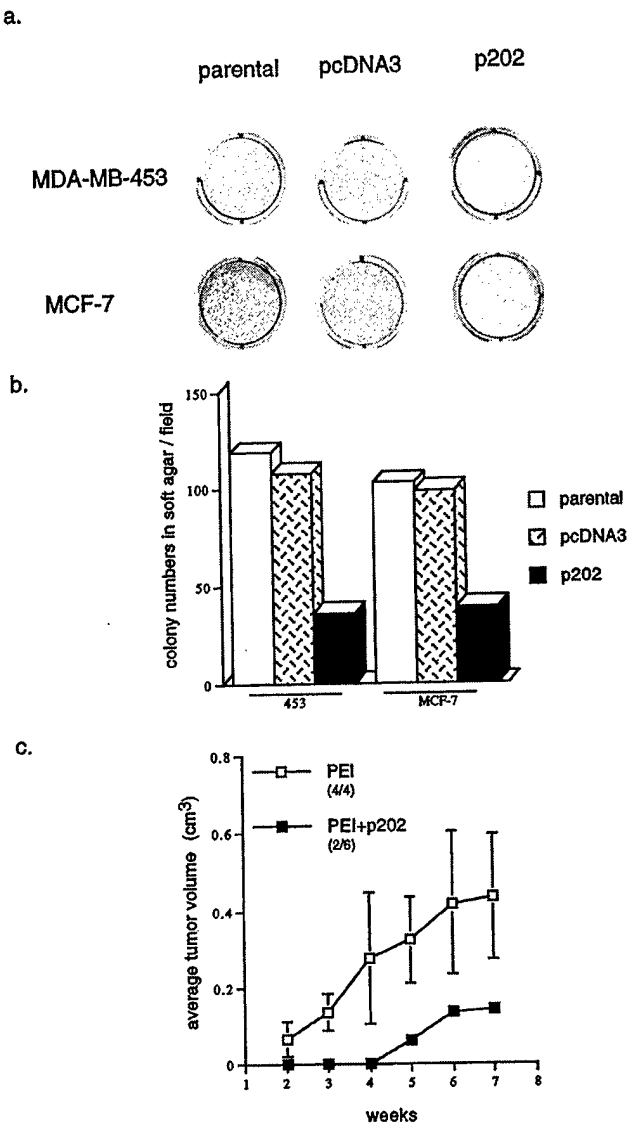

Fig. 2. p202 inhibits the transformation phenotype of breast cancer cells. *a*, colony formation in soft agar. MDA-MB-453, 453-pcDNA3, 453-p202, MCF-7, MCF-7-pcDNA3, and MCF-7-p202 cell lines were subjected to anchorage-independent growth in soft agar. *b*, number of colonies formed in soft agar as shown in *a*. The number represents the average of five random microscopic fields from each cell line. *c*, p202 *ex vivo* experiment. MCF-7 cells were transfected with p202 expression vector using PEI. After 24 h, cells were harvested, and the p202 (*PEI+p202*) or mock (*PEI*) transfected cells (3×10^6 cells/injection) were injected into the mammary fat pad of female nude mice. 17-β-Estradiol pellets were implanted s.c. into the mice 1-day before inoculation. Tumor formation was monitored every week. *Bars*, SE.

mor activity of p202, we performed an *ex vivo* tumorigenicity assay in an orthotopic breast cancer model. Briefly, CMV-p202/PEI or PEI alone (PEI is a polymer vector used for transfection) was transfected into MCF-7 cells before injection into the mammary fat pads of the estrogen-supplemented nude mice. The p202 transfection caused a drastic reduction of tumorigenesis of MCF-7 cells as compared with that of the mock transfection (PEI alone; Fig. 2*c*). Our data demonstrated, for the first time, that the p202 expression is associated with an antitumor activity in animals.

In an attempt to identify therapeutic agents that may cooperate with p202 to synergize the antitumor effect on breast cancer cells, we used FACS analysis (Fig. 3) to determine a potential synergism in inducing apoptosis. We found that the p202-expressing cells were more susceptible to TNF-α-induced apoptosis than the control cells, *i.e.*, after treatment with TNF-α (0, 10, 20 ng/ml) for 48 h, more 453-p202 cells were undergoing apoptosis (sub-G_1 population) than the parental 453 cells and 453-pcDNA3 control cells in a dose-dependent manner (Fig. 3*a*). Likewise, MCF-7-p202 cells were also found to be more sensitive to TNF-α-induced apoptosis than the parental MCF-7 cells in a dose-dependent manner (Fig. 3*b*). These results suggested that p202 expression could sensitize cells to TNF-α-induced apoptosis.

One possible mechanism of the p202-mediated sensitization to TNF-α-induced apoptosis is that p202 could antagonize the antiapoptotic function of NF-κB (13–15). To test that hypothesis, we tested whether p202 expression could affect the NF-κB-mediated transcription activation in response to TNF-α treatment. We cotransfected CMV-p202 and a NF-κB-activatable promoter-reporter construct (κB-luc), *i.e.*, an IκB promoter-driven luciferase gene, into 453 cells in the presence of TNF-α (Fig. 4*a*). As expected, κB-luc was readily activated in the presence of TNF-α. However, this TNF-α-induced transcription activation was repressed by p202 in a dose-dependent manner. To test whether p202 acted on the NF-κB molecule to elicit such transcription repression, we cotransfected CMV-p202 with a Rel-A (a p65 subunit of NF-κB) cDNA expression vector and κB-luc. As shown in Fig. 4*b*, whereas p202 expression alone has no effect on κB-luc, it could greatly repress NF-κB (Rel-A)-activated IκB pro-

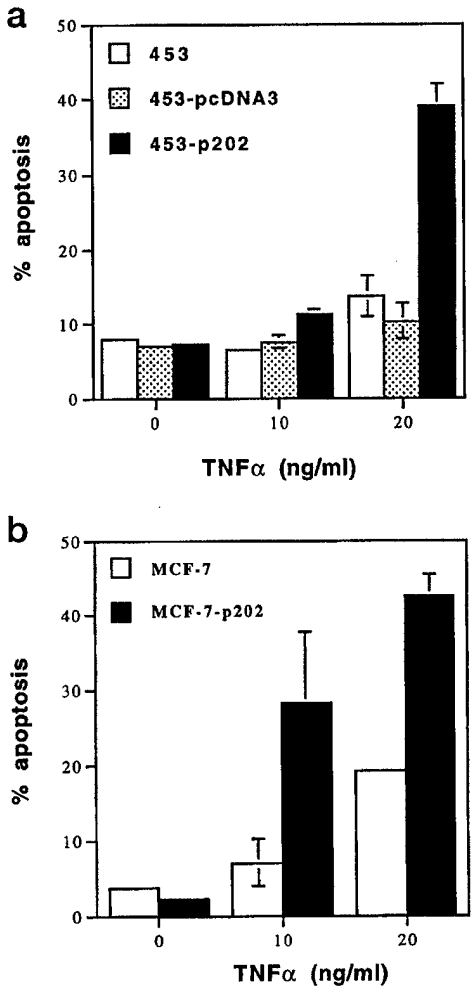

Fig. 3. p202 sensitizes breast cancer cells to apoptosis induced by TNF-α in a dose-dependent manner. *a*, 453, 453-pcDNA3, and 453-p202 cell lines were treated with TNF-α (0, 10, and 20 ng/ml) for 48 h. *Bars*, SE. *b*, MCF-7 and MCF-7-p202 were treated with TNF-α (0, 10, and 20 ng/ml) for 48 h. Cells were fixed and stained with propidium iodide. Apoptosis was quantitated by FACScan cytometer. *Bars*, SE.

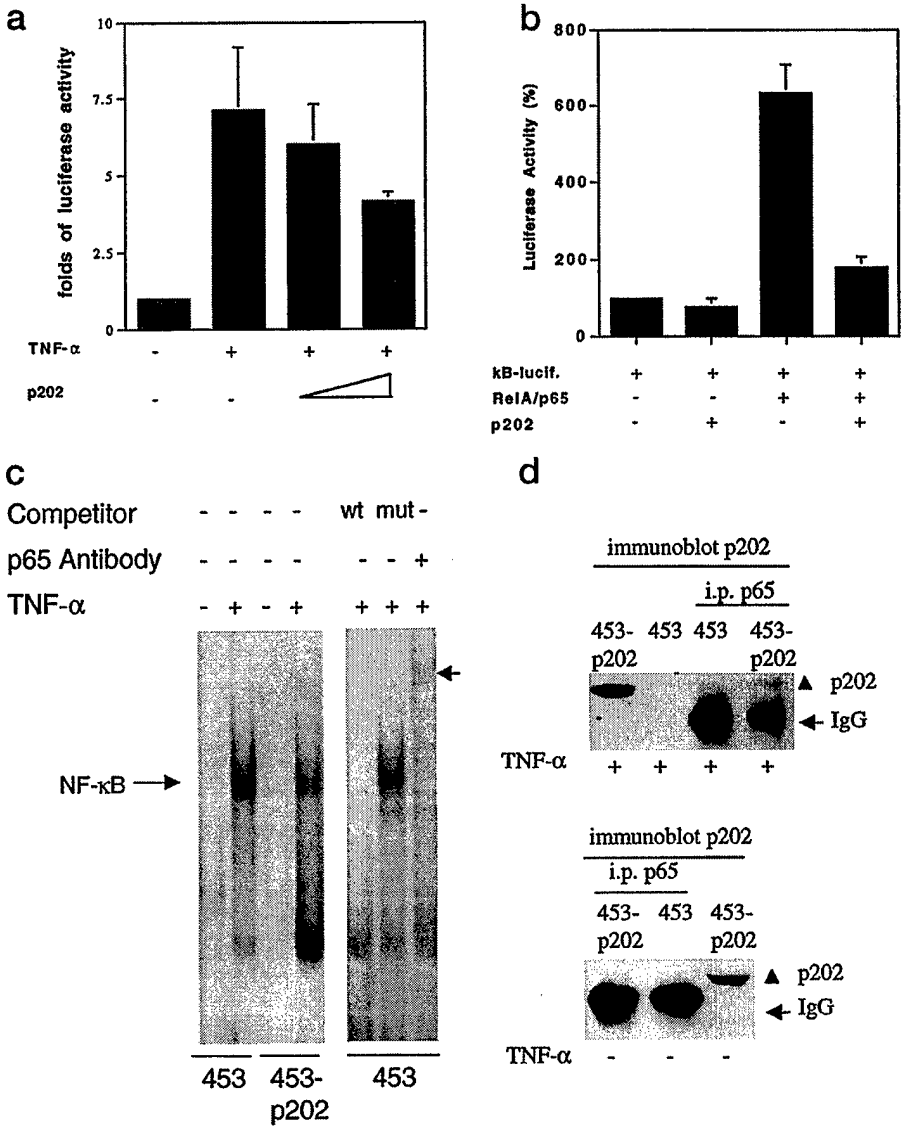

Fig. 4. The interaction and inactivation of NF-κB by p202 is responsible for the p202-mediated sensitization to TNF-α-induced apoptosis. *a,* p202 expression represses NF-κB-mediated transcription activation in response to TNF-α. IκB-Luciferase reporter gene (0.2 μg) and CMV-p202 (0, 0.8, or 2 μg) were cotransfected into MDA-MB-453 cells. Thirty-six h after transfection, cells were either left untreated or stimulated with TNF-α (20 ng/ml) for 6 h. The fold difference in IκB-Luciferase expression was calculated with respect to IκB-Luciferase expression in the absence of TNF-α and p202. *b,* p202 expression represses Rel-A (p65)-activated transcription. MDA-MB-453 cells were cotransfected with κB-luc and ± NF-κB (p65) expression vector. The inhibitory activity of p202 on the induction of IκB promoter activity by p65 was assessed by cotransfection with p202 expression vector. Luciferase activity was measured 48 h after transfection. The data represent an average of two independent experiments after normalization; *bars,* SE. *c,* gel-shift assay. 453 and 453-p202 nuclear extracts, used in this assay, were isolated from TNF-α-treated cells (20 ng/ml of for 30 min). *Left panel,* the activated NF-κB (p65/p50) induced by TNF-α is indicated by an *arrow. Right panel,* competition assay was performed in the presence of a 70-fold excess of wild-type or mutant oligonucleotides containing NF-κB binding site. A polyclonal Rel-A antibody supershifted the NF-κB complex to a slower-migrating position, as indicated by an *arrow. d, top panel,* p202 is physically associated with p65. 453 and 453-p202 cells were treated with or without TNF-α (20 ng/ml for 30 min). Cell lysates (1 mg) were used in the subsequent immunoprecipitation with anti-p65 antibody. Immunoprecipitated complexes were analyzed by SDS-PAGE, followed by immunoblotting with p202 antibody. *Bottom panel,* immunoblots of p202 protein using untreated 453 and 453-p202 cells serve as negative and positive controls, respectively. ▲, p202 band. IgG band is also indicated.

moter activity. These results suggest that the transcriptional repression of TNF-α-mediated gene expression by p202 may be attributable to the inactivation of NF-κB by p202.

This hypothesis was further supported by a subsequent observation that p202 expression was associated with a reduced level of the active NF-κB (p65/p50) molecule as measured by a gel-shift assay (Fig. 4*c, left panel*). As expected, the level of active NF-κB was found to be significantly increased in both the p202-expressing (435-p202) and the parental (453) cells treated with TNF-α (20 ng/ml). However, the level of activated NF-κB was greatly reduced in 453-p202. Using either a wild-type or mutant NF-κB DNA binding sequence as a competitor, we showed that the DNA/protein complex was indeed NF-κB specific in that only wild-type, but not mutant, sequence could compete with the NF-κB/DNA complex. Moreover, the fact that this complex could be supershifted in the presence of an anti-p65 antibody (Fig. 4*c, right panel*) further confirms the identity of this DNA/protein complex being NF-κB-specific. Thus, these data support the idea that p202 expression may impede the formation of active p65/p50 heterodimer. That, in turn, represses transcriptional activation induced by NF-κB.

It is possible that p202 may interact with p65, forming a p202/p65 complex, which may significantly reduce the concentration of free p65 in p202-expressing cells. To test that possibility, we performed a coimmunoprecipitation assay. As shown in Fig. 4*d, upper panel,* with TNF-α treatment, p202 could be coimmunoprecipitated with p65 by an anti-p65 antibody in 453-p202 nuclear extract but not 453 extract. As a control, no detectable p202 was observed in either cell line without TNF-α treatment (Fig. 4*d, lower panel*). These data strongly indicate that p202 and p65 are physically associated in the same complex upon TNF-α stimulation. The p65 protein level is comparable between 453 and 453-p202 cells with TNF-α treatment (data not shown), indicating that p202 may not regulate p65 expression.

The above observation presents a possible scenario that TNF-α-induced NF-κB activation could be antagonized by p202 via a p202/p65 interaction. That, in turn, causes subsequent transcriptional repression of genes, the activation of which requires active NF-κB. Although it has been reported previously that p202 could bind both p50 and p65 *in vitro* and p50 *in vivo* (6), our data are the first demonstration of an *in vivo* association between p202 and p65 upon TNF-α stimulation. Taken together, our results provide a possible mechanism that accounts for the p202-mediated sensitization to TNF-α-induced apoptosis in breast cancer cells.

Inflammatory cytokines, *e.g.,* TNF family members, can transduce apoptotic signals in certain tumor cells and have been tested in a number of clinical trials (16). Despite the promising data in animal models, unsatisfactory results have been observed in many clinical trials (17). It might be attributable to the resistance of many cancer cells to TNF-α-induced apoptosis, presumably, by the activation of NF-κB and the subsequent induction of survival factors that counteract apoptosis. In this report, we demonstrated that p202 expression not only exerted strong growth retardation and tumor suppression activities in breast cancer cells but also is able to sensitize these cells to TNF-α-induced apoptosis, and that sensitization is associated with inactivation of NF-κB via a p202/p65 interaction. Thus, our data implicate a potential therapeutic application of a combined treatment of TNF-α and p202 gene therapy for cancer patients.

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p202, an Interferon-inducible Protein, Mediates Multiple Antitumor Activities in Human Pancreatic Cancer Xenograft Models[1]

Yong Wen,[2] Duen-Hwa Yan,[2] Bailiang Wang, Bill Spohn, Yi Ding, Ruping Shao, Yiyu Zou, Keping Xie, and Mien-Chie Hung[3]

Departments of Molecular and Cellular Oncology [Y. W., D-H. Y., B. S., Y. D., R. S., Y. Z., M-C. H.], Surgical Oncology [D-H. Y., M-C. H.], Gastrointestinal Medical Oncology [B. W., K. X.], The University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

p202, an IFN-inducible protein, interacts with certain transcriptional activators leading to transcriptional repression. p202 expression has been associated with inhibition of cancer cell growth *in vitro* and *in vivo*. To examine a potential p202-mediated antitumor activity in pancreatic cancer, we used both ectopic and orthotopic xenograft models and demonstrated that p202 expression is associated with multiple antitumor activities that include inhibition of tumor growth, reduced tumorigenicity, prolonged survival, and remarkably, suppression of metastasis and angiogenesis. *In vitro* invasion assay also showed that p202-expressing pancreatic cancer cells are less invasive than those without p202 expression. That observation was supported by the findings that p202-expressing tumors showed reduced expression of angiogenic markers, such as interleukin 8 and vascular endothelial growth factor, and p202-expressing pancreatic cancer cells have reduced level of matrix metalloproteinase-2 activity, a secreted protease activity important for metastasis. Importantly, we demonstrated a treatment efficacy by using *p202*/SN2 liposome complex in a nude mice xenograft model, suggesting a feasibility of using the *p202*/SN2 liposome in future preclinical gene therapy experiments. Together, our results strongly suggest that p202 expression mediates multiple antitumor activities against pancreatic cancer and may provide a scientific basis for developing a *p202*-based gene therapy in pancreatic cancer treatment.

INTRODUCTION

Pancreatic cancer is highly aggressive and is a leading cause of cancer death in Western countries. The deadliness of this disease is illustrated by the prediction in 1999 that 28,600 new cases would be diagnosed and most of them would be fatal (1). The main reason for the extremely poor prognosis is the fact that patients often present with advanced stage at the time of diagnosis. The median survival varies between 4 and 6 months, and the 5-year survival rate is <2% (2). Currently, there is no effective treatment for this deadly disease because conventional chemotherapy and radiation treatments have had very limited success to improve patient survival (3). Therefore, novel treatment strategies against this disease are urgently needed.

p202 is an IFN-inducible protein, and its expression is associated with growth inhibition (4, 5). The findings that p202 interacts with cell cycle transcriptional regulators, such as E2F-1/DP-1, E2F-4/DP-1, AP-1 (c-Fos/c-Jun), and c-Myc, and represses their transcriptional activities have provided insight into the molecular mechanism by which p202 mediates growth inhibition (6–10). We have documented previously that p202 inhibits human cancer cell growth *in vitro* and suppresses tumor growth *in vivo* (11, 12). Furthermore, we showed that p202 expression sensitizes breast cancer cells to TNF-α-induced apoptosis. The mechanism responsible for the p202-mediated sensitization is likely attributable to the inactivation of TNF-α-induced NF-κB[4] by p202 (12). In light of the antiapoptotic role of NF-κB in TNF-α-mediated apoptosis (13), we have hypothesized that NF-κB inactivation by p202 leads to the abolishment of the antiapoptotic process and results in sensitizing cancer cells to TNF-α-induced apoptosis. On the other hand, the aberrant NF-κB activity has been implicated, at least in part, in tumorigenesis and the chemoresistant phenotype of certain human cancers including pancreatic cancer (14, 15). Thus, it is likely that *p202*-based gene therapy may be particularly useful in targeting tumors that contain such aberrant NF-κB activity. In this study, we tested that possibility in pancreatic cancer cells that possess the constitutively active NF-κB (16). We showed that, in addition to the growth inhibition *in vitro* and tumor suppression *in vivo*, p202 expression was found associated with suppression of metastasis and angiogenesis in an orthotopic pancreatic cancer xenograft model. Importantly, we demonstrated a treatment efficacy of using *p202*/liposome gene therapy in a pancreatic cancer xenograft model. Together, our results raise a possibility of using *p202*-based gene therapy strategy in pancreatic cancer treatment.

MATERIALS AND METHODS

Cell Culture. Human pancreatic cancer cell lines Capan-1, PANC-1, BxPC-3, AsPC-1, and CFPAC-1 were obtained from the American Type Culture Collection and maintained as recommended. Transfected cell lines were maintained in complete medium containing 500 μg/ml G418 (Life Technologies, Inc.).

Colony-forming Assay. Cells were transfected with a p202 expression vector (CMV-*p202*; in which *p202* cDNA is driven by CMV promoter) or an control vector (pcDNA3). Both plasmids contain the neomycin-resistant gene. Three weeks after transfection and G418 selection, cell colonies were stained by 0.5% crystal violet containing 20% ethanol.

Western Blot Analysis. Protein lysate was prepared with RIPA-B cell lysis buffer containing 20 mM Na_2PO_4 (pH 7.4), 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 2 mM Na_3VO_4, 5 mM phenylmethylsulfonyl fluoride, 1% aprotinin, and 10 μg/ml leupeptin. Rabbit anti-p202 polyclonal antibody was kindly provided by Dr. Divaker Choubey (Loyola University, Chicago, IL). Donkey antirabbit IgG peroxidase (Jackson) was used as secondary antibody. Western blots were developed by enhanced chemiluminescence (ECL; Amersham).

Northern Blot Analysis. Total RNA was isolated from PANC-1, pcDNA3, p202-1, and p202-2 cells using a TRIZOL RNA isolation kit (Life Technologies, Inc.). Twenty μg of total RNA were separated by electrophoresis under denaturing conditions and then transferred to a Hybond N^+ membrane. Full-length *p202* cDNA isolated from CMV-*p202* plasmid by *Bam*HI digest was gel-purified and ^{32}P-labeled by using a random-labeling kit. Hybridization was performed at 65°C overnight in solution containing 1% BSA (w/v), 0.2 M sodium phosphate, 1 mM EDTA, 7% SDS (w/v), 15% formamide, and 40

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[2] These authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Department of Molecular and Cellular Oncology, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3668; Fax: (713) 794-0209; E-mail: mchung@mdanderson.org.

[4] The abbreviations used are: NF-κB, nuclear factor-κB; CMV, cytomegalovirus; luc, luciferase; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; IL, interleukin.

Table 1 *Number of colonies from transfection of pancreatic cell lines with p202 and control vector DNA*

Cell line	pcDNA3	p202	% of control
Capan-1	130	3	2.3
PANC-1	508	108	21.0
BxPC-3	4	1	25.0
AsPC-1	232	31	13.4
CFPAC-1	6	0	0

μg/ml salmon testes DNA (Sigma Chemical Co.). The blot was subsequently washed three times in 40 mM sodium phosphate, 1 mM EDTA, 1% SDS (w/v) at 65°C for 5 min/wash and twice at 70°C for 10 min/wash. The *p202* RNA (1.8 kb) was visualized by using a PhosphorImager.

Transfection and luc Assays. PANC-1, pcDNA3, p202-1, and p202-2 cells were transfected with 0.5 μg of κB-luc construct and 0.1 μg of the internal transfection control (pRL-TK; Promega). Forty-eight h after transfection, cells were harvested, and luc activity was measured using the dual luc assay system (Promega) according to the protocol supplied by the manufacturer. The κB-luc activity was normalized by the internal control luc activity of pRL-TK. To determine the p202 dose effect, PANC-1 cells were cotransfected with 50 ng of CMV-luc and an increasing amount (0, 0.5, or 2 μg) of CMV-*p202*. The total amount of DNA transfected at each p202 dose was kept constant (2.05 μg) by adding an appropriate amount of pcDNA3 vector. luc activity was measured 48 h after transfection. The relative activities were calculated by setting the luc activities obtained from transfections without CMV-*p202* (0 μg) at 100%. The data represent mean ± SD of two independent experiments.

Soft Agar Assay. Aliquots of cells (1×10^4) were mixed at 37°C with 0.5% agarose (Sea Plaque, low gelling temperature; FMC Bioproducts, Rockland, ME) in complete medium and gelled at 4°C for 15 min over a previously gelled layer of 1% agarose in complete medium in six-well dishes. After incubation for 3 weeks, 200 μl of 1 mg/ml *p*-iodonitrotetrazolium violet were added and incubated for an additional 24 h. Colonies were photographed using a Zeiss microscope and counted using computer software associated with the microscope.

Ectopic Tumorigenicity Assays in Nude Mice. Aliquots of cells (1×10^6) in 200 μl of PBS were injected s.c. on both sides of the abdomen of female nude mice, 4–5 weeks of age. Tumor sizes were measured with a caliper every week. The tumor volume was calculated using the formula: Volume = $S \times S \times L/2$, where S is the short length of the tumor in cm and L is the long length of the tumor in cm.

Orthotopic Tumorigenicity and Survival Assays in Nude Mice. Aliquots of cells (1×10^6) were suspended in 50 μl of PBS as single-cell suspensions. Nude mice were anesthetized with methoxyflurane and placed in the supine position. An upper midline abdomen incision was made, and the pancreas was exteriorized. Tumor cells were injected into the tail of the pancreas, and the abdomen was closed using wound clips. Animals were sacrificed 3 months after tumor inoculation. Tumors in the pancreas were harvested and weighed. Livers were fixed in Bouin's solution for 24 h to differentiate the neoplastic lesions from the organ parenchyma, and the metastases on the surface of liver were counted with the aid of a dissecting microscope. For survival assays, daily survival of mice was monitored and recorded as dead or euthanized when the animals reached the moribund stage.

***In Vitro* Invasion Assay.** The procedure was followed as described previously (17), except for the following modification. The 24-well chamber with an 8-μm pore size polycarbonate filter (Costar Co., Cambridge, MA) was coated with Matrigel (Becton Dickinson Labware, Bedford, MA) according to the manufacturer's protocol.

Zymography. Cells were grown to 70% confluency in DMEM/F12 medium containing 10% FBS and switched to serum-free medium (17). After 2 days of incubation, the conditioned medium was collected, passed through a 0.22 μm filter, and then concentrated to a small volume on Centricon YM 30 filter units. Four μg of each sample were loaded on the gel. For the positive control, 0.25 μl of FCS was used. Zymography was performed using gelatin-embedded SDS gels as described previously (17).

Immunohistochemistry. Tumor tissue sections (5 μm thick) of the formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene and rehydrated in graded alcohol. The endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The samples were incubated for 20 min at room temperature with a protein blocking solution containing 5% normal horse serum and 1% normal goat serum and in PBS. Samples were then incubated at 4°C in a 1:50 dilution of rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA) or 1:50 dilution of rabbit anti-VEGF antibody, followed by the incubation with peroxidase-conjugated antirabbit IgG at room temperature for 1 h, and with diaminobenzidene (Research Genetics) for 5 min. The sections were counterstained with Mayer's hematoxylin (Biogenex Laboratories, San Ramon, CA) and mounted with a Universal mount (Research Genetics). When examined under a microscope, a positive reaction was indicated by a reddish-brown precipitate in the cytoplasm or the nucleus. Tissue sections vessels in solid tumors growing in the pancreas of nude mice were determined under light microscope after immune staining of sections with anti-CD31 antibodies. Cryostat sections of tumors were fixed with 2% paraformaldehyde in PBS (pH 7.5) for 10 min at room temperature and processed for immunostaining as described above for paraffin-embedded tissues.

***p202* Gene Therapy Treatment in a Human Pancreatic Cancer Xenograft Model.** Aliquots of PANC-1 cells (1×10^6) in 200 μl of PBS were injected (per site) s.c. on both sides of the abdomen of female nude mice, 4–5 weeks of age. After tumors reached 5 mm in diameter, mice received treatment with the *p202*/SN2 complex through intratumor injection. For each injection, 15 μg of CMV-*p202* or the control vector, *i.e.*, a luc cDNA driven by a CMV promoter (CMV-*luc*), was complexed with 30 μg of SN2. CMV-*p202*/SN2

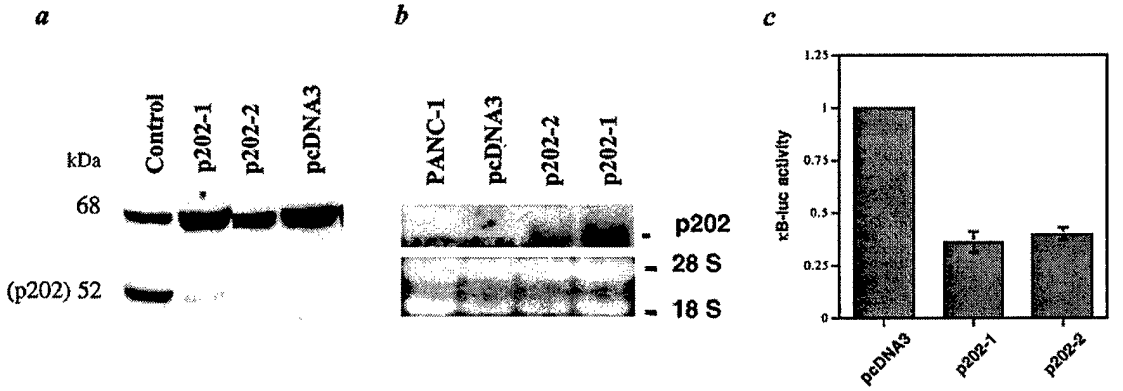

Fig. 1. Generation of the p202-expressing PANC-1 cells. *a*, two p202-expressing PANC-1 cell lines (p202-1 and p202-2) were generated, and p202 protein (M_r 52,000) expression was analyzed by Western blot using p202-specific antibody. The M_r 68,000 nonspecific band serves as an internal loading control. The positive control was AKR-2B cells stimulated by IFN-α (*Control*), and the vector (*pcDNA3*)-transfected cells served as negative control. *b*, p202 mRNA expression in p202-1 and p202-2 cells. Twenty μg of total RNA isolated from PANC-1, pcDNA3, p202-1, and p202-2 cells were analyzed by Northern blot using a full-length p202 cDNA as a probe. The p202-specific RNA (1.8 kb) is indicated. As internal loading controls, 18S and 28S rRNAs on the membrane after gel transfer were stained by ethidium bromide. *c*, p202 expression inhibits the NF-κB-mediated transcription. A *luc* gene driven by IκB promoter (*κB-luc*) was transfected into p202-1, p202-2, and vector control (*pcDNA3*) cells, followed by luc assay. The relative luc activity is shown with that in pcDNA3 cells set at 100%. *Bars*, SD.

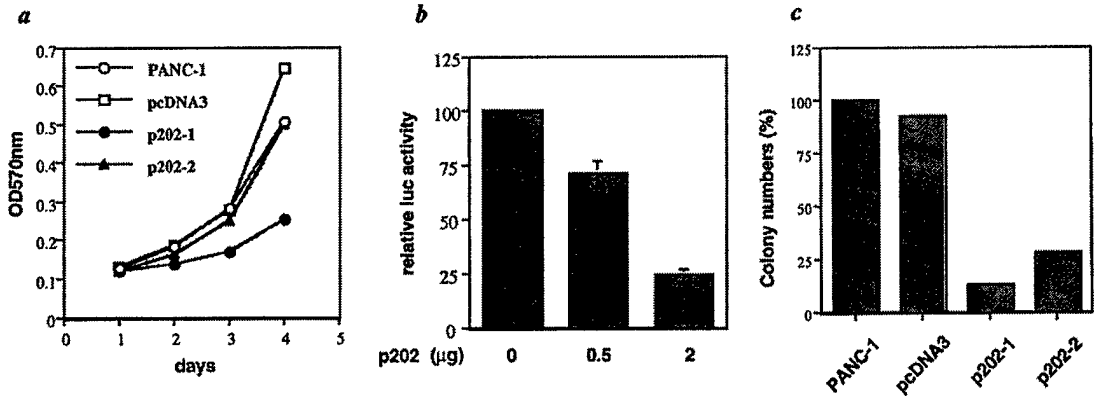

Fig. 2. The p202-expressing PANC-1 cells exhibit a reduced growth *in vitro*. *a*, the growth rate of PANC-1 (parental), pcDNA3, p202-1, and p202-2 cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Each measurement was done in quadruplicates. *b*, the p202 dose effect on growth inhibition. PANC-1 cells were cotransfected with 50 ng of CMV-luc and an increasing amount (0, 0.5, or 2 μg) of CMV-p202. The relative activities were calculated by setting the luc activities obtained from transfections without CMV-p202 (0 μg) at 100%. The data represent means of two independent experiments; *bars*, SD. *c*, PANC-1, pcDNA3, p202-1, and p202-2 cells were grown in soft agar. The colony number was scored 3 weeks after seeding by *p*-iodonitrotetrazolium violet staining. The relative number is presented using that of the parental cells as 100%.

complex was administrated twice a week. Mice in the control group were injected with either CMV-*luc*/SN2 complex or SN2 alone. Tumor size and treatment related side effect were monitored twice a week. SN2 is a liposome-based, nonviral delivery system developed recently by our group.[5]

RESULTS

p202 Expression Inhibits Human Pancreatic Cancer Cell Growth *in Vitro*. To determine the growth-inhibitory activity of p202 in human pancreatic cancer cells, we performed a colony-forming assay on five human pancreatic cancer cell lines, *i.e.*, Capan-1, PANC-1, BxPC-3, AsPC-1, and CFPAC-1. [All of them are known to possess the constitutively active NF-κB (16).] As shown in Table 1, with all cell lines tested, the number of G418-resistant colonies in the *p202*-transfected cells was consistently fewer than that of the pcDNA3-transfected cells with a reduction ranged from 75% (BxPC-3) to 100% (CFPAC-1). This result suggests that p202 possesses a strong growth-inhibitory activity in human pancreatic cancer cells. Consistent with that observation, our effort to isolate p202 stable pancreatic cancer cell lines has yielded only 2 p202-expressing PANC-1 clones (*i.e.*, p202-1 and p202-2) of 20 G418-resistant clones screened by Western analysis. p202-1 expresses a higher level of p202 protein than p202-2 (Fig. 1*a*), and that correlates well with the levels of *p202* mRNA expression of these clones as determined by a Northern blot analysis (Fig. 1*b*). To test whether p202 expression inhibits NF-κB activity in the p202-expressing cells (12), we performed a gel-shift assay and found that the p202 expression level in p202-1 and p202-2 cells is efficient to abolish the DNA binding activity of NF-κB (data not shown). The reduced NF-κB DNA binding activity in p202-expressing cells was further confirmed by the reduced NF-κB-mediated promoter activity in p202-1 and p202-2 cells as compared with that in the control cells (pcDNA3; Fig. 1*c*). As shown in Fig. 2*a*, the growth rate of p202-1 cells is the slowest as compared with that of PANC-1, vector control (pcDNA3), and the low p202 expressor, p202-2 cells. The different levels of p202 expression may account for the different growth rates seen between p202-1 and p202-2 cells. This result is consistent with our previous observation that the extent of growth inhibition is p202 dose dependent (11). To further confirm that observation, we performed a transient transfection assay in which a fixed amount (50 ng) of CMV-*luc* was cotransfected with an increasing amount (0, 0.5, and 2 μg) of CMV-*p202* in PANC-1 cells. Because the apparent luc activity is indicative of living cells, we showed that p202 expression caused overall growth inhibition in a dose-dependent manner (Fig. 2*b*). Under the same condition, no apparent apoptosis was observed as determined by flow cytometry analysis to detect sub-G_1 apoptotic cells (data not shown). Thus, our

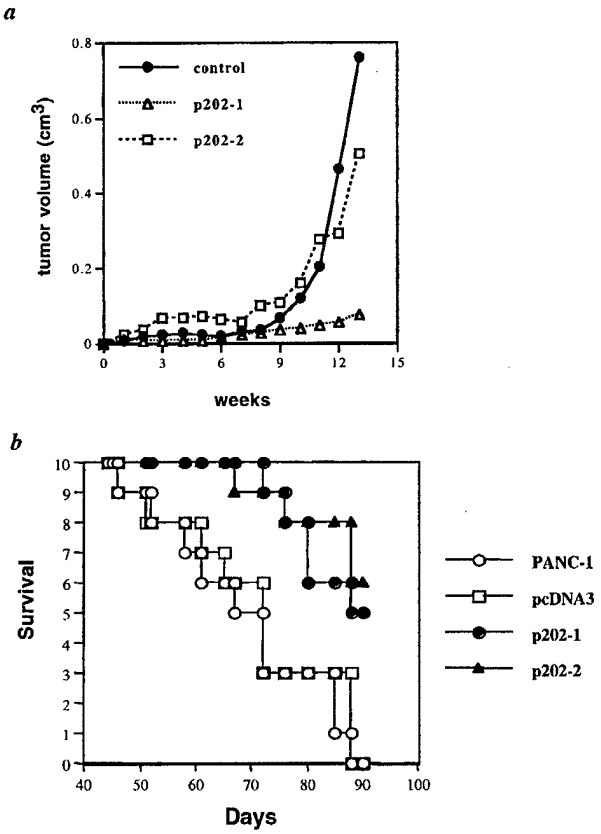

Fig. 3. p202 expression mediates antitumor activity *in vivo*. *a*, tumorigenicity assay. PANC-1 (*control*), p202-1, and p202-2 cells were s.c. implanted in the abdomen of 4–5-week-old female nude mice (five mice/group). Tumor sizes were measured with a caliper every week. The tumor volume was calculated using the formula: Volume = $S \times S \times L/2$, where S is the short length of the tumor in cm and L is the long length of the tumor in cm. *b*, mice bearing p202-expressing PANC-1 tumors in pancreas exhibited a longer survival rate. PANC-1, pcDNA-3, p202-1, and p202-2 cells were injected orthotopically into mouse pancreases. The time of death was recorded, and the survival rate was calculated as the percentage of the surviving animals (the starting animal number was set at 100%).

[5] Y. Zou and M-C. Hung, unpublished results.

Table 2 *Growth and metastasis of PANC-1 human adenocarcinoma cells in nude mice*[a]

	Orthotopic growth (pancreas)		Gross hepatic metastasis	
Lines	Incidence	Median (tumor weight, g)	Incidence	Median (number)
PANC-1	5/5	0.30 (0.12, 0.20, 0.30, 0.51, 0.63)	2/5[b]	0 (0, 0, 0, 3, 4)
pcDNA3	5/5	0.22 (0.08, 0.13, 0.22, 0.32, 0.46)	1/5[c]	0 (0, 0, 0, 0, 5)
p202-1	1/5	0 (0, 0, 0, 0, 0.06)[d]	0/5	0 (all 0)[d]
p202-2	2/5	0 (0, 0, 0, 0.045, 0.05)[d]	0/5[e]	0 (all 0)[d]

[a] Othortopic tumor transplantation (1×10^6 cells/mouse) was performed by intrapancreatic injection. Mice were killed after 3 months.
[b] Two mice were found with peritoneal dissemination.
[c] One mouse was found with peritoneal dissemination.
[d] $P < 0.01$ as compared with control groups.
[e] One mouse was found dead before the experiment terminated with no tumor and no metastasis.

results support the idea that the level of p202 expression is proportional to its growth-inhibitory activity. Consistent with the ability of p202 to suppress transformation phenotype (11, 12), both p202-1 and p202-2 cells showed a significant reduction in the number of soft-agar colonies as compared with that of the control cell lines, *i.e.,* PANC-1 and pcDNA3 (Fig. 2*c*). Our results thus indicate that p202 is a potent growth inhibitor in suppressing pancreatic cancer cell growth.

p202 Expression Suppresses Tumorigenicity in Ectopic and Orthotopic Pancreatic Cancer Xenograft Models. To determine whether p202 expression could mediate an antitumor effect on pancreatic cancer cells *in vivo*, we first examined whether p202 suppresses the tumorigenicity of PANC-1 cells. p202-1 and p202-2 cells were s.c. injected into nude mice ,and tumor growth was monitored thereafter. As shown in Fig. 3*a*, p202-1 tumors grew significantly slower than the vector-transfected PANC-1 tumors. p202-2 tumors (which express less p202 than p202-1 tumors) on the other hand started to show a modest growth reduction 12 weeks after injection as compared with that of the control tumors. Similar to p202-mediated growth inhibition, the extent of antitumor activity observed here appears to be dependent on the level of p202 expression. To further examine the p202-mediated antitumor activity in an organ (pancreas)-specific environment, we injected p202-1 and p202-2 cells directly into mouse pancreas. Three months after injection, mice were sacrificed, and tumor growth was measured. We found that although PANC-1 tumors and vector control tumors grew readily in mouse pancreas at 100% frequency (five of five), p202-1 and p202-2 cells are tumorigenic at a much lower frequency, *i.e.,* 20% (one of five) and 40% (two of five), respectively (Table 2). Furthermore, the average tumor size (measured by weight) of the control tumors was about five times that of p202-1 or p202-2 tumors. When the survival rate was measured, we observed that mice bearing p202 tumors had a longer survival than those bearing control tumors without p202 expression (Fig. 3*b*). In particular, mice bearing p202-1 or p202-2 tumors had 50% survival at 90 days after implantation as opposed to the mice bearing either PANC-1 or vector control tumors that showed 0% survival at the same time. Our results clearly demonstrated a potent antitumor activity of p202 in orthotopic pancreatic cancer xenograft models but to a lesser extent in the ectopic environment.

p202 Expression Suppresses Metastasis in Pancreatic Tumors. Upon examining the orthotopic pancreatic tumor xenograft model, we observed liver metastasis in 40% (two of five) and 20% (one of five) of mice bearing either PANC-1 tumors or vector control tumors, respectively. In contrast, we found no detectable liver metastasis in mice bearing p202 tumors (Table 2). This result suggests a possible antimetastasis function of p202 in pancreatic cancer cells. To test that possibility *in vitro*, we used a double-chamber assay (17) in which the test cells were grown in the top chamber, and the bottom chamber was

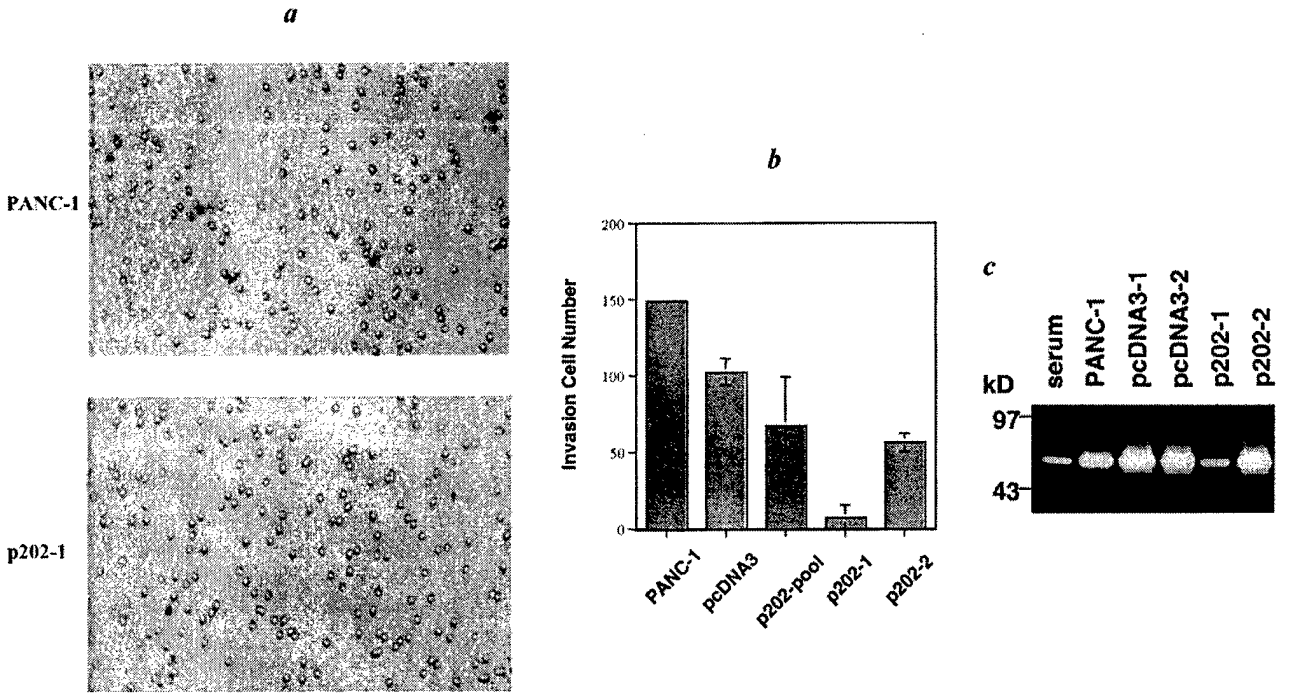

Fig. 4. p202 expression inhibits cell invasion *in vitro*. Cells were tested for their invasiveness by using a double-chamber assay in which a Matrigel-coated membrane was used to separate two chambers. PANC-1, pcDNA3, a pool of p202-transfected clones (p202-pool), p202-1, and p202-2 cells were seeded in the top chamber with lower chamber filled with laminin (30 μg/ml)-containing conditioned medium. Seventy-two h after seeding, the cells that had migrated on the bottom side of the membrane were scored by Giemsa's stain, as indicated by the *blue* cells (*a*); the quantification of the cells that migrated is shown *b*. *Bars,* SD. *c*, p202 expression is associated with a reduced level of secretory MMP-2 in the medium. Cells [PANC-1, two vector-transfected cell lines (pcDNA3-1 and pcDNA3-2), p202-1, and p202-2] were grown in the serum-free medium. The secreted MMP-2 activity was assayed by zymography. After 24 h incubation, the conditioned medium was harvested and concentrated before being subjected to SDS-PAGE, in which the gel was imbedded with 1.5% gelatin. The MMP-2 activity in serum served as a positive control.

filled with conditioned medium containing a chemoattractant, *e.g.*, laminin. A Matrigel-coated membrane was used to separate the two chambers. To migrate from the top chamber to the bottom chamber, cells must digest away the reconstituted basement membrane matrix by producing secretory proteases, such as MMPs, and then penetrate through pores on the membrane. Thus, this assay somewhat mimics a typical metastatic process, and the number of cells found on the bottom side of the membrane [which can be visualized by Giemsa staining, *i.e.*, blue cells (Fig. 4*a*)] is indicative of the metastatic potential of the test cells. On the basis of these criteria, we found that p202-1 possesses the least metastatic potential among the cell lines tested (Fig. 4*b*). p202-2 and p202-pool cells (*i.e.*, the pooled *p202*-transfected clones) have slightly lower metastatic potential than that of PANC-1 or vector control cells (Fig. 4, *a* and *b*). Because MMP-2 (M_r 72,000) is one of the important MMPs secreted by cancer cells during the metastatic process (18), we examined whether MMP-2 expression is altered in p202-1 and p202-2 cells by using a zymography to analyze the MMP-2 activity in each cultured medium. As shown in Fig. 4*c*, the level of MMP-2 secreted by p202-1 cells is greatly reduced, but PANC-1, vector control (pcDNA3-1 and pcDNA3-2), and p202-2 cells maintain a high level of secreted MMP-2. Although the MMP-2 level is not significantly reduced in p202-2 cells, it is likely that the low p202 expression level accounts for the difference in both the MMP-2 activity and the *in vitro* invasiveness between p202-1 and p202-2 cells. The MMP-2 activity in serum serves as a positive control. Together, our *in vivo* and *in vitro* results support the idea that p202 expression suppresses metastatic potential of pancreatic cancer cells.

p202 Expression Suppresses Angiogenesis in Pancreatic Tumors. It has been well documented that tumor growth and metastasis require persistent growth of new blood vessel (neovasculature; Ref. 19). To examine whether the reduced tumorigenicity of p202-expressing pancreatic cancer cells is associated with a reduced angiogenesis, we analyzed the formation of neovasculature in p202-1 tumors and PANC-1 tumors obtained from the orthotopic pancreatic cancer xenografts. Fig. 5 shows that the number of blood vessels [stained by antibody against a blood vessel marker, *i.e.*, CD31 (20)] was significantly reduced in p202-1 tumor as compared with PANC-1 tumor. Because the expression of angiogenic factors such as IL-8 and VEGF are critical for the onset of angiogenesis (19), we examined a possible correlation between the expression of these proteins and the reduced angiogenesis in p202-1 tumors. Using immunohistochemical analysis with antibody specific to IL-8 or VEGF, we showed that p202-1 tumor has much reduced IL-8 and VEGF protein staining (dark gray color) as compared with that of PANC-1 tumor (Fig. 5). These results strongly suggest that p202 expression in pancreatic tumors is associated with suppression of angiogenesis.

***p202*/Liposome Treatment Suppresses Tumor Growth in a Pancreatic Cancer Xenograft Model.** On the basis of the strong antitumor activity of p202 in human pancreatic cancer cells described above (Fig. 2 and Table 2), we tested a potential therapeutic effect of *p202* gene therapy treatment in a s.c. pancreatic cancer model. Briefly, the mice bearing s.c. PANC-1 tumors were treated by intratumor injection of CMV-*p202*/SN2 complex twice a week for 8 weeks. (SN2 is a lipid formula developed in our laboratory, and when complexed with DNA, it enhances *in vivo* and *in vitro* transfection efficiency.[5]) The control groups consisted of tumor-bearing mice treated with SN2 alone (SN2) or CMV-*luc*/SN2 complex. As shown in Fig. 6, although there was no significant difference in tumor growth between SN2 and CMV-*luc*/SN2 complex treatment groups, the CMV-*p202*/SN2-treated tumors exhibited a slower growth rate than that of the control treatments. This proof-of-concept experiment clearly shows a feasibility of using CMV-*p202*/SN2 complex to achieve a therapeutic effect on an ectopic pancreatic cancer xenograft model.

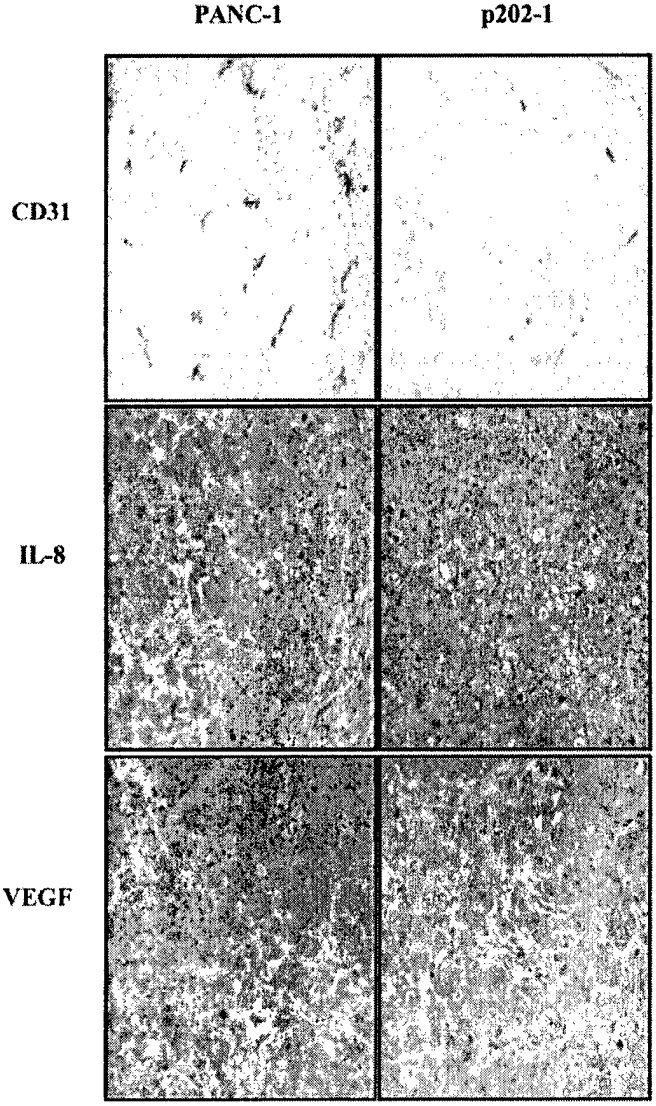

Fig. 5. p202 expression is associated with reduced angiogenesis and down-regulation of IL-8 and VEGF. PANC-1 and p202-1 tumors excised from the pancreas of the orthotopic xenografts were analyzed by immunohistochemical staining for vascularity (CD31 staining) and IL-8 and VEGF protein expression.

DISCUSSION

In this report, we showed that p202 expression resulted in a growth inhibition of pancreatic cancer cells *in vitro* and *in vivo*. The enforced expression of p202 correlates well with the inactivation of the otherwise constitutively active NF-κB. This observation is significant because persistent NF-κB activity was associated with antiapoptosis and chemoresistance in human cancers (14, 15), and that sets the stage for us to test whether p202 expression can sensitize these pancreatic cancer cells to chemotherapy treatment. To date, very few chemodrugs are found beneficial in treating pancreatic cancer (3), and the constitutive activation of NF-κB is likely to contribute to such chemoresistant phenotype. It is therefore possible that a *p202*-based gene therapy may restore chemosensitivity and lead to a potential *p202*/chemo-drug combined treatment for pancreatic cancer. Experiments are under way to test this hypothesis.

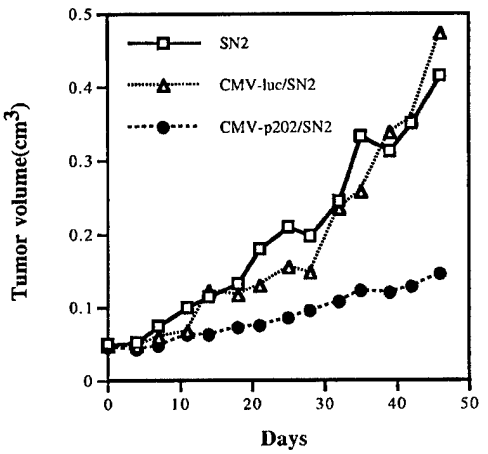

Fig. 6. Antitumor effect of p202/SN2 complex by intratumoral injection in a s.c. xenograft model. Tumors were produced by s.c. implanting PANC-1 cells into both flanks of each nude mouse. Tumor-bearing mice were divided into three treatment groups (five mice/group and two tumors/mouse): SN2 liposome alone (*SN2*), *CMV-luc/SN2*, and *CMV-p202/SN2*. SN2 (30 μg) with or without DNA (15 μg) in 100 μl of PBS was injected twice a week into each tumor. Tumors were measured twice a week after treatment began, and the average tumor volume per treatment group at the indicated time is presented.

We observed a more pronounced p202-mediated antitumor activity (*i.e.*, reduced tumor growth and prolonged survival) in the orthotopic pancreatic cancer xenograft model than that in a s.c. ectopic xenograft model. Although the reason for the differential therapeutic effect remains unknown, it does indicate that the orthotopic xenograft model is not only a more relevant but also a better model to evaluate future treatment efficacy of *p202*-based gene therapy than the s.c. ectopic model. We also observed a decreased vascularity (CD31 staining) and a decreased level of angiogenic factors, *e.g.*, IL-8 and VEGF, in p202-expressing tumors. Thus, our results suggest that p202 inhibits the expression of angiogenic factors and that in turn leads to suppression of angiogenesis. Remarkably, we observed that mice bearing p202-expressing tumors apparently lacked liver metastasis, and this result correlates with a reduced invasiveness and a reduction in MMP-2 expression *in vitro*. Together, our data present the first evidence to suggest that p202 expression is associated with suppression of both angiogenesis and metastasis.

Finally, we showed a significant therapeutic effect using CMV-*p202*/SN2 treatment in an s.c. pancreatic cancer xenograft model. It nevertheless implicates a feasibility of using a *p202*-based gene therapy treatment for pancreatic cancer. Given that the orthotopic model is a better system to study the efficacy of p202 antitumor activity as indicated in this report, it is possible that CMV-*p202*/SN2 treatment may yield even better antitumor activity in an orthotopic xenograft model. Success of a *p202*-based gene therapy in orthotopic model will pave the way for future experiments to determine the efficacy *p202*/chemo-drug combined treatment in preclinical gene therapy settings.

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Advances in Brief

Systemic Tumor Suppression by the Proapoptotic Gene *bik*[1]

Yiyu Zou, Hua Peng, Binhua Zhou, Yong Wen, Shao-Chun Wang, Eing-Mei Tsai, and Mien-Chie Hung[2]

Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

Metastatic breast cancer requires systemic treatment. We have developed a systemic gene therapy approach for breast cancer, consisting of a nonviral gene delivery system (SN) and a proapoptotic gene, *bik*. The transfection efficiency of SN carrying a reporter gene was 5–10 times higher than the common nonviral agents Fugene-6 and Lipofectamine in the presence of serum. The SN-*bik* gene complex induced significant apoptosis in four breast cancer cell lines *in vitro* as well as in orthotopic tumor tissues in nude mice. Systemically administrated SN-*bik* significantly inhibited the growth and metastasis of human breast cancer cells implanted in nude mice and prolonged the life span of the treated animals. This study demonstrates that SN-*bik* is a promising approach for further development as a potential therapeutic agent of cancer.

Introduction

The *bik* gene is a proapoptotic member in the Bcl-2 gene family. Loss of informative alleles on chromosome 22q where the *bik* gene is located may be related to the development of human breast and colorectal cancers (1, 2). The *bik* gene encodes a M_r 18,000 protein product, which contains a BH3 domain critical for its proapoptotic activities (3, 4). *bik* forms heterodimers with various antiapoptotic proteins, including Bcl-2 and Bcl-X_L (3, 4), the association of which hinders the function of the antiapoptotic proteins. *bik* triggers apoptosis through a *p53*-independent pathway (4), suggesting a broad potential of *bik* to target different types of cancer cells. However, a method for delivering the *bik* gene to human cells *in vivo* has not been reported.

Breast cancer is a metastatic disease. Drug delivery systems able to systemically target cancer cells are required for efficient treatment of breast cancer patients. Nonviral gene delivery systems have been attractive strategies for therapeutic application. Cationic lipids of high binding affinity with negatively charged DNA molecules are the most commonly used vehicle because of their minimal immunogenicity and low toxicity *in vivo*. However, the low DNA transduction efficiency and instability in the presence of serum has limited the application of lipid-based systemic delivery systems. In this report, we used a novel formulation of cationic lipid that resulted in enhanced biostability and tumor suppression function when used to deliver the proapoptotic *bik* gene to breast tumor cells *in vitro* and *in vivo*.

Materials and Methods

Cell Lines. Human breast cancer cell lines MCF7, MDA-MB-231, MDA-MB-435, and MDA-MB-468 and the human non-small cell lung carcinoma cell line A549 were purchased from the American Type Culture Collection (Rockville, MD) and cultured according to the vendor's instructions.

Expression Plasmids. bik, luc[3]-, p53-, and GFP-expressing plasmids were constructed by inserting the cDNAs of *bik*, *luc*, *p53*, and *GFP*, respectively, into the pcDNA3 vector containing a cytomegalovirus promoter.

Formulation. The gene delivery system, termed SN, was essentially a cationic liposome formulation composed of dipalmitoylethylphosphocholine, dioleoylphosphoethanolamine, dipalmitoylphospho-ethanoamine, and polyethyleneglycol. The DNA was entrapped in the liposome using the thin-lipid film hydration method and extrusion through a filter with 0.2-μm-diameter pores (Gelman Sciences; Ann Arbor, MI) as described previously (5). The liposomal DNA particles were 60–170 nm in diameter.

Transfection. Cells were cultured for 24 h in six-well plates with 1 ml/well of DMEM/F12 medium with 10% FBS (Life Technologies, Inc., Gaithersburg, MD) until 60–70% confluence was reached. The liposomal DNA (SN-DNA or Lipofectamine-DNA complex) or nonliposomal DNA (Fugene-6-DNA complex) was directly added into the culture plates at a ratio of 2 μg of DNA/10^6 cells. Twenty-four h later, the transfection efficiency was determined by counting the *GFP*-positive cells under a fluorescence microscope and expressing the result as a percentage of total cells. Six random fields with >200 cells/field were counted for each sample. All experiments were repeated three times independently.

Colony Formation in Soft Agarose. The standard colony formation assay (6) was used to test whether transfection of *bik* in cationic liposomes inhibits colony formation of tumor cells in soft agarose. Briefly, cells of the human breast cancer lines MDA-MB-231 and MDA-MB-468 were transfected with SN-DNA. One day after the transfection, the cells (5×10^3 cells/well) were plated in six-well plates in culture medium containing 0.5% agarose overlying a 1% agarose bottom layer and cultured at 37°C with 5% CO_2. Five weeks later, the top layer of the culture was stained with *p*-iodonitrotetrazolium (1 mg/ml). Colonies >100 μm in diameter were counted.

Apoptosis Assay. For *in vitro* studies, standard fluorescence-activated cell sorter analysis was used to determine the apoptosis of the cells. Briefly, the cells were transfected with SN-*bik* or other agents. Forty h after transfection, the apoptotic cells were assessed by flow cytometric detection of sub-G_1 DNA content after being stained with propidium iodide. Fields with >2000 cells in each were randomly selected, and the apoptotic *versus* nonapoptotic cells were counted. For *in vivo* studies, female nude mice (*nu/nu*, 7–8 weeks of age, 18–22 g; Harlan Sprague Dawley, Madison, WI) were inoculated with the human breast cancer cell line MDA-MB-231 in the MFPs at a dose of 2×10^6 cells/mouse (one tumor/mouse). Five weeks later, the tumor-bearing mice were randomly divided into two groups with three mice in each group. The mice in the treatment group received a single i.v. injection of SN-*bik*, 15 μg of DNA/mouse. The mice in control groups received the same dose of SN-*luc*. One day after the injection, the tumors and other organs were resected. The tissues were fixed in 10% buffered formalin for 12 h and were then processed and embedded in paraffin. The slides were then deparaffinized, rehydrated, refixed in 4% formalin, and digested in 20 μg/ml proteinase K solution for 15 min. The slides were washed in PBS and refixed in 4% formalin, washed again in PBS, and equilibrated in equilibration buffer. Biotinylated nucleotide mix and TdT enzyme were added and incubated for 1 h at 37°C; slides were washed in PBS, blocked in hydrogen peroxide, and incubated in streptavidin horseradish peroxidase. The slides were developed in 3,3′-diaminobenzidine and counterstained with Harris hematoxylin. The apoptotic cells (brown staining) were counted under a microscope. The apoptosis index was defined by the percentage of brown cells among the total cells of each sample. Ten fields with >200 cells in each were randomly counted for each sample.

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[2] To whom requests for reprints should be addressed, at Department of Molecular and Cellular Oncology, Box 108, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3668; Fax: (713) 794-0209; E-mail: mhung@mail.mdanderson.org.

[3] The abbreviations used are: luc, luciferase; GFP, green fluorescent protein; FBS, fetal bovine serum; MFP, mammary fat pad; RLU, relative luc unit; RES, reticuloendothelial system.

***Ex vivo* Tumor Inhibition.** MDA-MB-231 and MDA-MB-468 cells were transfected by SN-*bik* or SN-*luc*. Twenty-four h after transfection, the cells were carefully trypsinized, harvested, and inoculated into the MFPs of nude mice (2×10^6 cells/tumor). The volume of the resulting tumor was measured weekly.

Tissue Distribution of the SN-delivered Reporter Gene. MDA-MB-231 cells were inoculated into MFPs of the nude mice. Five weeks later, the tumor-bearing mice received a single i.v. injection of SN-*luc* at a dose of 60 μg/mouse. One day after the injection, the mice were killed, and the tumors and other organs were resected and immediately frozen on dry ice. The tissues were homogenized after adding 1× lysis buffer (Promega Corp., Madison, WI) with a volume (μl) equivalent to five times the tissue weight (mg). The tissue suspension was centrifuged at 2500 × *g* for 10 min after undergoing a freeze-thaw procedure. *luc* activity in the supernatant was determined with a luminometer (Promega). The *luc* activity in 100 mg of tissue was used to compare gene expression in different tissues. To compare the gene delivery capabilities, the commercial transfection kits Fugene-6 and Lipofectamine were used as controls. The carrier:DNA ratio was 2:1 for SN-*luc*, 5:1 for Fugene-6-*luc*, and 9:1 for Lipofectamine-*luc* complex.

Antitumor Activity Tests. To study tumor growth inhibition, female nude mice were inoculated with 2×10^6 of breast cancer cells/tumor into the MFPs. Two weeks later, when most tumors exceeded 4 × 4 mm, the tumor-bearing mice were randomly divided into three groups with 5 mice in each group. The mice in all treatment groups received i.v. injections of SN-*bik* twice a week for 3 weeks, at a dose of 15 μg of DNA/mouse. The mice in control groups were injected with the same dose of SN-*luc* or the same volume of PBS. The tumor volume was measured weekly. To assess animal survival and the increase in life span, the same tumor models and the same therapeutic treatments were used. The experiment was terminated on day 200 after tumor inoculation. To evaluate tumor metastasis, the nude mice inoculated with 2×10^6 MDA-MB-468 cells into their MFPs were randomly divided into three groups with 5 mice in each group. Two weeks after the inoculation, the mice were treated by SN-*bik*, SN-*luc*, or PBS as mentioned above. Autopsies were performed on sacrificed mice at week 10. The metastasis was identified in the peritoneal cavity, and the numbers of metastatic tumor nodules <1 mm in diameter were counted.

Statistical Analysis. All statistical tests used in this study are two-sided log-rank statistical tests.

Results

SN Efficiently Transfected Genes into Cancer Cells in Serum-supplemented Cultures. The formulated SN liposome was first tested for its gene delivery efficiency by transfection of a reporter gene, *GFP*, in cell culture. To mimic the systemic administration condition, transfection was performed in medium supplemented with serum. For comparison, SN transfection was done side by side with Fugene-6 and Lipofectamine, the most popular commercial transfection agents. Four different breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-468) were transfected in the presence of 10% FBS. The transfection efficiency of SN was 12–32-fold higher than that of Lipofectamine in all cell lines tested ($P < 0.001$; Fig. 1*A*). SN was 1.6–1.8-fold more efficient in MCF7, MDA-MB-231, and MDA-MB-468 cells ($P < 0.01$) and similar ($P > 0.05$) in MDA-MB-435 cells compared with Fugene-6. SN and the commercial transfection kits showed similar transfection efficiencies in serum-free cultures (data not shown).

SN Efficiently Delivered Gene to Orthotopic Breast Tumors in Mice via i.v. Injection. We further tested the gene delivery capability of SN by determining the tissue distribution after tail vein injection in nude mice. Nude mice with MDA-MB-231-derived tumors inoculated in the MFPs were given tail-vein injections of luciferase gene (60 μg) entrapped in SN (SN-*luc*). Twenty-four h after injection, mice were killed, protein extracts isolated from the tumors and different organs, and the relative luc activity was determined using a luminometer. For comparison, Fugene-6 and Lipofectamine were also included in the test. The results showed that overall SN was more efficient in systemic gene delivery than Fugene-6 (Fig. 1*B*). The luciferase expression levels in SN-*luc*-injected mice were 13-fold higher in tumors and 2–7-fold higher in normal organs compared with Fugene-6-*luc*-injected mice ($P < 0.001$). There was no detectable luciferase signal

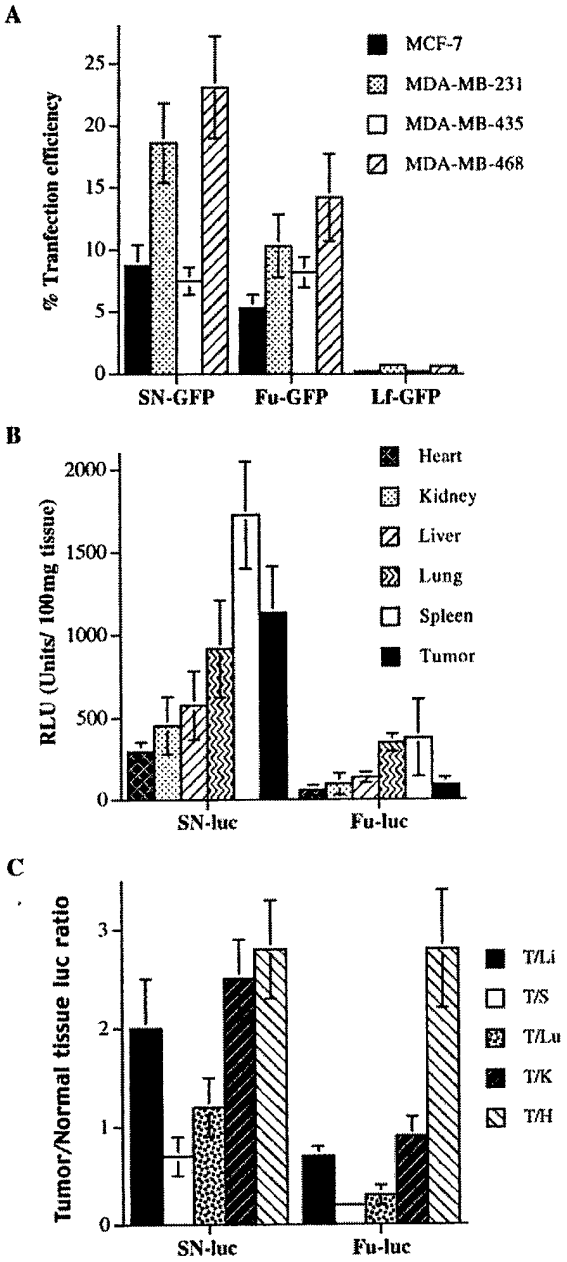

Fig. 1. SN is a better nonviral gene delivery system in serum-containing tissue culture. *A*, human breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-435, or MDA-MB-468 ($4-5 \times 10^5$ cells/well in six-well plates) were transfected with SN-encapsulated *GFP* (SN-*GFP*), Fugene-6-*GFP* complex (Fu-*GFP*), or Lipofectamine-*GFP* complex (Lf-*GFP*), respectively, in DMEM/F12 medium supplemented with 10% FBS. The transfection condition was optimized for each formulation. The transfection efficiency was determined by counting the percentage of fluorescent cells under a fluorescence microscope. Each sample was randomly counted in six fields with >200 cells/field. The data are the means from three independent experiments; *bars*, SD. *B*, SN is efficient for systemic gene delivery to breast tumors *in vivo*. MDA-MB-231 cells (2×10^6 cells/mouse) were inoculated into the MFPs of nude mice. Six weeks later, mice bearing tumors received single i.v. injections of SN-entrapped luciferase plasmid (SN-*luc*). Twenty-four h after the injection, the luc activities in tumor and normal tissues as indicated were determined by a luminometer. The luc activity in 100 mg of wet tissue was used as RLUs to measure gene expression and distribution in tissues. The commercial transfection kit Fugene-6 was used as a control. The data are the means from three mice of each group; *bars*, SD. The tumor:tissue expression ratios of luciferase are summarized in *C*. *Bars*, SD.

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in any organ and tumor in Lipofectamine-*luc*-injected mice (data not shown). By comparing the tumor *versus* normal tissue ratio of *luc* activity, we found that SN-mediated gene delivery was more tumor specific, and the tumor:normal tissue ratio of SN-*luc*-injected mice was 2–3-fold higher than that of Fugene-6-*luc*-injected mice (Fig. 1*C*). The luciferase expression level in the orthotopic tumors of SN-*luc*-injected mice was higher than in all other tissues except spleen. The RLUs in the tumors were 2.0-, 2.5-, and 3.8-fold higher than that in livers, kidneys, and hearts ($P < 0.01$, $n = 3$), respectively, and it was similar to that in lung (1132 to 915 RLU; $P > 0.01$). The higher gene expression in tumors implied the possibility that i.v. injection of SN encapsulating a therapeutic gene would kill breast tumor cells without life-threatening toxicity.

***bik* Transfection Induced Significant Apoptosis of Cancer Cells *in Vitro*.** Having shown the efficiency and specificity of the SN lipid in DNA delivery, we next tested the apoptosis induction of SN lipid combined with the *bik* gene. Breast cancer cell lines MCF-7, MDA-MB-435, MDA-MB-231, and MDA-MB-468 were transfected by either a *bik*- or a *luc*-expressing plasmid entrapped in the SN lipid (SN-*bik* or SN-*luc*) in serum-supplemented medium, and the levels of apoptotic cells were determined by flow cytometry (Fig. 2*A*). The results indicated that SN efficiently introduced the apoptotic gene into various cancer cell lines and resulted in a remarkable rate of apoptosis induction. The induction of apoptosis was independent of the status of the *p53* gene in the cancer cells because cell lines with mutated p53 (MDA-MB-231 and MDA-MB-468) or wild-type p53 (MCF-7 and A549) were similarly affected by the treatment. In contrast, SN-*p53* had only a minimal effect on A549 and MCF-7, which contain wild-type *p53* gene (7).

The SN-*bik*-induced apoptosis resulted in reduced potential for colony growth of cancer cells in soft agar. Compared with the non-treated control, colony formation of the SN-*bik*-treated MDA-MB-231 and MDA-MB-468 cells was reduced 86 and 95%, respectively, whereas only a minor effect was observed in the SN-vector-treated cells (Fig. 2*B*). These results suggest that SN-*bik* transfection can inhibit tumorigenicity of breast cancer cells.

SN-*bik* Inhibited Tumor Growth in Nude Mice. The antitumor activity of SN-*bik* was next tested in an *ex vivo* assay. The MDA-MB-231 and MDA-MB-468 cells were transfected in cell culture by SN-*bik* or SN-*luc*, and the transfected cells were then inoculated into the MFPs of nude mice. The growth of tumors was followed weekly (Fig. 3). SN-*bik* delayed tumor growth in mice by at least 3 weeks compared with the *luc* control. The tumor volume ratios of control *versus* treatment groups during weeks 2 to 9 ranged from 1.6 to 8.0 for MDA-MB-231 and from 1.6 to 6.9 for MDA-MB-468, suggesting a strong tumor suppression activity by SN-*bik* treatment *in vivo*.

We used an orthotopic breast cancer model to confirm the tumor suppression activity of SN-*bik*. MDA-MB-231 and MDA-MB-468 cells were inoculated into the MFPs of nude mice. Mice with established tumors were then treated with SN-*bik*, SN-*luc*, or PBS through tail vein injection. SN-*bik* injection significantly inhibited tumor growth in mice compared with the PBS- or SN-*luc*-treated mice. By week 5, the mean tumor volume of PBS- and SN-*luc*-treated mice was ~3-fold higher than that of SN-*bik*-treated mice in the MDA-MB-231 model ($P < 0.001$; Fig. 4*A*); in the MDA-MB-468 model, the mean tumor volume of control mice was 2-fold higher than that of SN-*bik*-treated mice ($P < 0.001$; Fig. 4*B*). The most significant tumor suppression effect could be observed by week 10, with an ~4-fold difference in tumor volumes between the control and treatment groups. In addition to decreasing tumor growth, SN-*bik* systemic treatment also strongly inhibited metastasis in nude mice as shown by a separate metastasis assay (Fig. 4*C*), suggesting that there are multiple mechanisms associated with the tumor suppression function of

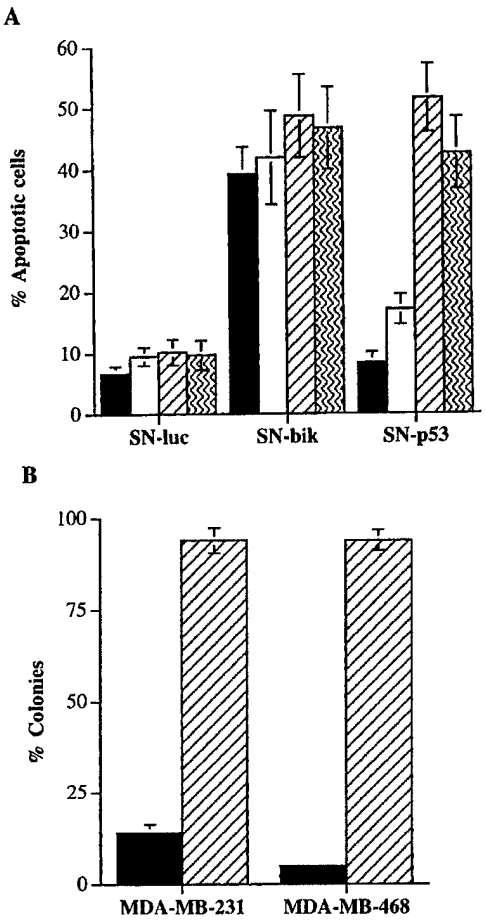

Fig. 2. Inhibition of cell proliferation by SN-delivered *bik*. *A*, the apoptotic potency of *bik* was compared with p53 in the human lung cancer cell line A549 (wild-type p53) and human breast cancer cell lines MDA-MB-231 (mutant p53), MDA-MB-468 (mutant p53), and MCF-7 (wild-type p53). The cells were transfected with SN-*bik* with 2 μg of DNA/10^6 cells in 10% serum containing medium. SN-*luc*-transfected cells were used as control. The apoptotic cells were determined with flow cytometry 24 h after transfection. ■, A549 (wt-p53); ▨, MDA-MB-231; □, MCF-7 (wt-p53); ▩, MDA-MB-468. *B*, MDA-MB-231 and MDA-MB-468 cells were transfected with SN-*bik* and seeded in 0.5% agar solution at 37°C in a six-well plate with 5×10^3 cells/well. The cells transfected with SN-entrapped vector (SN-vector) were used as controls. The number of cell colonies at least 100 μm in diameter was counted 4 weeks later. The ratios of colony number of the treated cells to the colony number of control cells are shown. The data are the averages from three independent duplicate experiments; *bars*, SD. ■, SN-bik; ▨, SN-vector.

bik. The potential of clinical application of SN-*bik* was assessed in a gene therapy setting using an MDA-MB-468-derived orthotopic breast cancer model. Systemic treatment by SN-*bik* significantly increased the survival rate of the treated mice compared with the control groups treated with PBS or SN-*luc* ($P < 0.001$; Fig. 4*D*).

We have examined tumor tissues after i.v. SN-*bik* injection. A significant amount of apoptotic cells (17% ± 3.5%) was detected in the orthotopic breast tumor tissue after single i.v. injection of SN-*bik*. In contrast, fewer apoptotic cells (2.7% ± 0.8%) could be identified in the liver tissue of the same animal (Fig. 4, *E* and *F*). SN-*luc* injection did not induce a significant level of apoptosis in tumor or liver tissues. These results suggest that treatment by the particular liposome-*bik* formulation can lead to significant tumor cell death *in vivo* with low, nonspecific cytotoxicity in other normal tissues.

Discussion

We have shown that *bik* gene expression delivered by SN resulted in enhanced apoptosis and tumor suppression of breast cancer cells. In the soft agar and apoptosis assays, we observed more dramatic growth

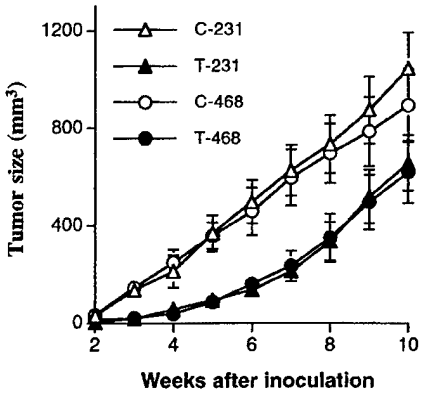

Fig. 3. *Ex vivo* assay of SN-*bik* tumor suppression function. Cells (2×10^6) from the MDA-MB-231 and MDA-MB-468 lines were transfected with SN-*bik* (*T*) or SN-*luc* (*C*) in culture plates. Twenty-four h later, treated cells were inoculated in the MFPs of nude mice, with 5 mice in each group. Tumor size was measured weekly (*triangles*, MDA-MB-232; *circles*, MDA-MB-468).

inhibition effects than would be expected based on the data of transfection efficiency. A possible explanation is that the expression of apoptotic genes, such as *bik*, can result in significant bystander effects, as described previously by others (8–10). In addition, the nature of low sensitivity of *GFP* scoring, which was used to determine the transfection efficiency of the SN lipid, may have underestimated the transfection efficiency.

The *bik* gene is a potent inducer for apoptosis. Apoptosis triggered by *bik* does not require the function of *p53* (4). This was also shown in this study in which *bik* induced apoptosis in a panel of cancer cell lines, regardless the status of the *p53* gene (Fig. 2, *A* and *B*). This property makes *bik* a potentially useful anticancer agent against cancers that do not respond to *p53* or p53-dependent gene therapy. As potent as it is, the effectiveness of *bik* depends on its systemic targeting to cancer cells, which requires a delivery system that can withstand i.v. conditions and carry the therapeutic gene to the targeted cells.

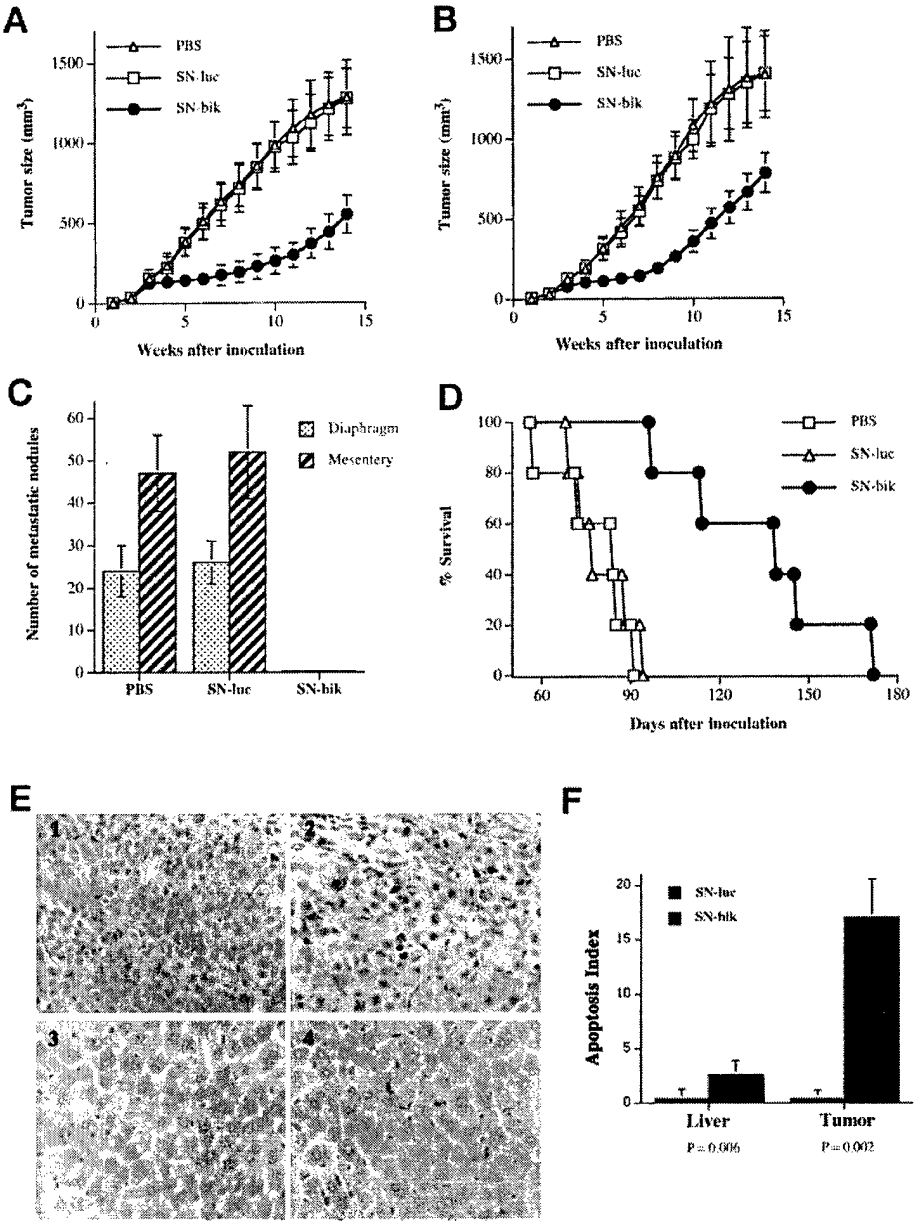

Fig. 4. The *bik* gene delivered by SN significantly inhibited growth of orthotopic breast tumors in mice. *A* and *B*, human breast cancer cell lines MDA-MB-231 (*A*) and MDA-MB-468 (*B*) were inoculated into the MFPs of nude mice at 2×10^6 cells/tumor. After 2 weeks, the mice bearing tumors were randomly divided into three groups with 5 mice in each. One group received six i.v. injections of SN-*bik* (15 μg DNA), one group received SN-*luc* (15 μg DNA), and one group received the same volume of PBS, all with a 3-day interval. *C*, i.v. injection of SN-*bik* inhibited metastasis of orthotopic breast cancer in mice. MDA-MB-468 cells were inoculated into the MFPs of nude mice, and the mice bearing tumors were treated with SN-*bik*, SN-*luc*, or the same volume of PBS as described in *A* and *B*. The treatment started on day 21 after inoculation, and the mice were sacrificed on day 60. Metastatic tumor nodules with diameter >1 mm in the peritoneal cavity were counted. *D*, the *bik* gene delivered by SN significantly prolonged the life of mice with orthotopic breast cancer. MDA-MB-468 cells (3×10^6 cells/site) were inoculated into the MFPs of nude mice. After 3 weeks, the tumor-bearing mice were randomly divided into three groups with 5 mice in each and treated as described in *A* and *B*. *E*, systemic i.v. SN-*bik* treatment induced a high level of apoptotic cells in the tumor tissue but not in normal organs. The tumor (*1* and *2*) and liver (*3* and *4*) tissue samples from SN-*bik* (*2* and *4*) or SN-*luc* (*1* and *3*) injected mice were used for *in vivo* apoptosis assay. Apoptotic cells (*brown* staining) were determined based on terminal deoxynucleotidyl transferase-mediated nick end labeling assay as described in "Materials and Methods." *F*, the numbers of apoptotic cells in liver and tumor tissues were counted and compared between SN-*bik* and SN-*luc* treatments. *Bars*, SD.

Nonviral gene delivery by liposomes is a promising strategy because the liposome vehicle has very low immunogenicity and toxicity. The clinical application of liposomal treatment is, however, haunted by the low stability and the low DNA transduction efficiency in the presence of serum, a condition encountered with systemic treatment. The main reason is that, structurally, these formulations are simply formed by complexing the liposome particles with DNA by the opposite static charge. Thus, DNA, liposome, and the liposome-DNA complexes are all exposed to neutralizing serum components and the RES directly, without protection. Once any one of these elements in the formulation is destroyed or inactivated, gene delivery is aborted. That is why liposome- and peptide-DNA complexes, such as Lipofectamine and Fugene-6, are inefficient *in vivo*, and most of the complexed particles quickly disappear from blood within several minutes after i.v. injection (11–13). In this study, we used a modified cationic liposome formulation (SN) containing a surface-protection polymer to stabilize the liposome-DNA particles for i.v. injection. This formulation entraps condensed DNA into the internal aqueous phase of the liposomes, and the positively charged liposome surface is coated by the polymers to protect the liposome from the attack of serum components, *e.g.,* high-density lipoprotein and the RES. The particle size of SN-DNA is also very small, ranging from 60 to 170 nm. Liposome stabilization by entrapment and surface protection make the SN formulation a more efficient i.v. DNA delivery system than other common nonviral gene delivery systems, such as Fugene-6 and Lipofectamine. An assay of the organ distribution showed higher tumor *versus* normal tissue ratios after i.v. injection of SN-*luc* than of Fugene-6-*luc* (Fig. 1, *B* and *C*), suggesting that the RES, which typically clears such particles from the circulation, engulfed SN-DNA particles at a slower rate than the other nonviral gene delivery systems tested.

Our results showed that the SN formulation significantly enhanced the expression index of the targeting gene in the tumor tissue. More importantly, the SN system supported efficient i.v. delivery of the therapeutic gene. This characteristic makes the SN formulation an attractive system to target metastatic disease systemically. A combination of SN and a therapeutic gene such as the proapoptotic *bik* gene, under the control of a tissue/tumor-specific promoter, would further increase the tumor targeting index. Once that is accomplished, titration of doses and administration schedules to achieve the best therapeutic window would be warranted.

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17th Annual Scientific Meeting of the Society for Biological Therapy, November 7–10, 2002, Hilton La Jolla Torrey Pines, San Diego, CA. Contact: Chloe Tolzmann, Society for Biological Therapy, 611 East Wells Street, Milwaukee, WI 53202. Phone: 414.271.2456; Fax: 414.276.3349; E-mail: ctolzmann@socbiother.com; Website: www.socbiother.com.

International Symposium on Taxanes, November 19–21, 2002, Hotel Palace Royal, Quebec City, Canada. Contact: Dr. Michael Pagé, Taxane Symposium, Faculty of Medicine, Université Laval, Ste Foy, Quebec G1K 4C5, Canada. Phone: 418.656.2664; Fax: 418.656.7666; E-mail: bcm2@videotron.ca; Website: www.taxanes.com.

6th International Mesothelioma Conference, December 1–3, 2002, Perth, Western Australia. Contact: Maree Brannegan. E-mail: branigam@cyllene.uwa.edu.au; Website: http://www.imig.org.

18th World Congress of Digestive Surgery and 9th Hong Kong International Cancer Congress, December 8–11, 2002, Hong Kong Convention and Exhibition Centre, Wanchai, Hong Kong. Contact: Congress Secretariat, 18th World Congress of Digestive Surgery (WCDS2002), Department of Surgery, University of Hong Kong Medical Centre, Queen Mary Hospital, Hong Kong. Phone: 852.2818.0232/852.2855.4235; Fax: 852.2818.1186; E-mail: wcds2002@hkucc.hku.hk; Website: http://www.wcds2002.org.

Cancer of the Esophagus and Gastric Cardia: From Gene to Cure, December 13–15, 2002, Amsterdam, The Netherlands. Contact: European Cancer Centre, PO Box 9236, NL-1006 AE Amsterdam, The Netherlands. Phone: 31.(0)20.346.2547; Fax: 31.(0)20.346.2525; E-mail: ecc@ikca.nl; Website: www.EurCanCen.org.

Correction

In the article by Y. Zou *et al.*, entitled "Systemic Tumor Suppression by the Proapoptotic Gene *bik*," which appeared in the January 1, 2002 issue of *Cancer Research* (pp. 8–12), the formulation for the gene delivery system described in the Materials and Methods section on page 8 appeared incorrectly as, "The gene delivery system, termed SN, was essentially a cationic liposome formulation composed of **dipalmitoylethylphosphocholine, dioleoylphosphoethanolamine, dipalmitoylphospho-ethanoamine, and polyethyleneglycol.** The DNA was entrapped in the liposome **using the thin-lipid film hydration method and extrusion** through a filter with 0.2-μm-diameter pores (Gelman Sciences, Ann Arbor, MI) as described previously (5)." The correct formulation is "**1,2-dipalmitoyl-sn-glycerol-3-ethylphosphocholine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanoamine-N-polyethyleneglycol-5000, and polyethylenimine.** The DNA was entrapped in the liposome **after the thin lipid film was hydrated and extruded** through a filter with 0.2-μm-diameter pores (Gelman Sciences, Ann Arbor, MI) as described previously (5)."

Yiyu Zou
Hua Peng
Binhua Zhou
Yong Wen
Shao-Chun Wang
Eing-Mei Tsai
Mien-Chie Hung

Appendix 3

Editorial Manager(tm) for Cancer Cell
Manuscript Draft

Manuscript Number:

Title: Emodin Downregulates Androgen Receptor and Inhibits Prostate Cancer Cell Growth

Article Type: Research Article

Section/Category:

Keywords: emodin, prostate cancer, androgen receptor, degradation

Corresponding Author: Mien-Chie Hung

First Author: Tai-Lung Cha, M.D.

Order of Authors: Tai-Lung Cha, M.D.; Lin Qiu, Ph.D.; Yong Wen, M.D. Ph.D.; Mien-Chie Hung, Ph.D.

Abstract: The androgen receptor (AR) mediates androgen's effect on the initiation and progression of prostate cancer. Even in androgen-independent prostate cancer, AR still retains its function contributing to disease progression. Here we demonstrate that emodin, a natural compound, can directly target AR to suppress prostate cancer cell growth in vitro and prolong the survival of C3(1)/SV40 transgenic mice in vivo. Emodin treatment resulted in repressing androgen-dependent transactivation of AR by inhibiting AR nuclear translocation. Emodin decreased the association of AR and heat shock protein 90 and increased the association of AR and MDM2, which in turn, induces AR degradation in a ligand independent manner. Thus, emodin, through targeting AR may serve as a novel therapeutic and preventive agent for prostate cancers.

*** Cover Letter**

Mien-Chie Hung, Ph.D.
Director, Breast Cancer Basic Research Program
Ruth Legett Jones Chair
Chair, Dept. of Molecular and Cellular Oncology, Box 108
The University of Texas M. D. Anderson Cancer Center
Houston, TX 77030
Tel: (713) 792-3668 Fax: (713) 794-0209
Email: mhung@mdanderson.org

May 4, 2004

Editors, Cancer Cell
Cell Press
1100 Massachusetts Ave
Cambridge, MA 02138
Email: cancer@cell.com
Phone: 617 397 2829
Fax: 617 397 2810

Dear Editor:

Attached please find for our manuscript entitled "Emodin downregulates androgen receptor and inhibits prostate cancer cell growth" which we would like to ask your candid opinion to see whether Cancer Cell will be interested in reviewing the manuscript.

Prostate cancer has tremendously devastating impact on public health. Chemoprevention is considered to be particularly important to reduce incidence of prostate cancer because of its diagnosis in elderly men, and even a modest delay in the neoplastic development could result in a substantial reduction in the incidence of this clinical detectable disease. However, so far no effective chemopreventive agents are available. Recently, the result from a large Prostate Cancer Prevention Trial (PCPT) has caused tremendous attention [N Engl J Med, vol 349(3), 2003.215-224](TIME, July 7, 2003). In this trial which involved nearly 19,000 patients, finasteride, a 5-α reductase inhibitor was demonstrated to be effective on prevention of prostate cancer. However, finasterdie also increases the incidence of high-grade cancer which has high risk of death. This dilemma situation is attributed to the fact that finasteride reduces

androgen level and favors growth of androgen-independent prostate cancer [N Engl J Med, vol 349(3), 2003. 297-299]. Thus, a preventive agent directly targeting androgen receptor is considered to serve as a more effective agent to prevent prostate cancer. In this present study, we found that emodin, a natural compound, can downregulate AR both *in vitro* cell culture system and *in vivo* transgenic mouse model. A novel mechanism was also identified for emodin-mediated AR repression which involves sequential events effecting on AR transcriptional activity and protein stability. While studying the potential effect of emodin on prostate cancer prevention, we made the following interesting observation:

(1) Emodin inhibits prostate cancer cells proliferation especially AR-positive cells.
(2) Emodin inhibits AR transcriptional activity through preventing AR nuclear transloction.
(3) Emodin decreases association of AR and hsp90 and increases association of AR and MDM2 which in turn induces AR degradation through proteasome-mediated pathway in a ligand independent manner.
(4) Emodin prolongs the survival of C3(1)/SV40 transgenic mice attributed to downregulating AR and preventing disease progression, tumor invasion and maintaining body weight gain and physical activity.

Hormone (androgen-deprivation) therapies have not only been applied for the **treatment** but also for the **prevention** of prostate cancer. However, androgen-ablation-induced prostate cancer regression is usually transient and ends with regrowth of tumors that have become **androgen independent**. More and more evidence suggests that hormone therapies for prostate cancer might actually promote the more aggressive phenotype. Although the precise molecular mechanisms of androgen-independent prostate cancer are diverse and unclear, a **functional AR** is maintained during this stage. Our current study provides a novel mechanism of **emodin targeting AR itself rather than its ligands by inhibiting AR transcriptional activity and inducing AR degradation which prevents prostate cancer development and avoids the aggressive phenotype provoked by deprivation of androgens.** We believe that this finding should be of general interest to the readers of *Cancel Cell*.

We would like to recommend the following experts as candidate reviewers of this manuscript:

Timothy Thompson, Professor
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030
Work No. (713) 799-8718
Fax No. (713) 799-8712
Email: timothyt@bcm.tmc.edu

Leland Chung, Professor and Director
Emory University School of Medicine
1365 Clifton Rd. N. E. Suite B-4100
Atlanta, GA 30322-4710
Work No. (404) 778-4319
Email: lwchung@emory.edu

Lucia R. Languino
Professor
University of Massachuestts Medical School
Department of Cancer Biology
LRB, Rm 419
364 Plantation St.
Worcester, MA 01605
(T) 508-856-1606
(F) 508-856-1310
email: Lucia.Languino@umassmed.edu

Chawnshang Chang
George H. Whipple Professor
University of Rochester
Department of Pathology-Box 626
601 Elmwood Ave,
Rochester, NY 14642
(T) 585-275-9994
(F) 585-756-4133
email: Chang@URMC.Rochester.edu

Fazlul H Sarkar
Professor
Karmanos Cancer Institute,, Wayne State University School of Medicine
Department of Pathology
715 Hudson Webber Cancer Research Center,
110E Warren, Detroit, MI 48201
(T) 313-966-7279
(F) 313-966-7558
email: fsarkar@med.wayne.edu

Hsing-Jien Kung, Deputy Director
UC Davis Cancer Center
Res. Bldg. III, UCDMC
4645 2nd Avenue
Sacramento, CA 95817
Work No. (916) 734-1538
Fax No. (916) 734-2589
Email: hkung@ucdavis.edu

Thank you for your consideration for the possible review of our manuscript. We look forward to hearing from you soon.

With my best wishes!

Sincerely yours,

Mien-Chie Hung, Ph.D.
Professor and Chairman

Emodin downregulates Androgen Receptor and Inhibits Prostate Cancer Cell Growth

Tai-Lung Cha, Lin Qiu, Yong Wen, and Mien-Chie Hung

Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, and Graduate School of Biomedical Science, UT. Health Science Center, 1515 Holcombe Blvd, Houston, Texas 77030 USA. Correspondence should be addressed to M.-C.H. (mhung@mdanderson.org).

Summary

The androgen receptor (AR) mediates androgen's effect on the initiation and progression of prostate cancer. Even in androgen-independent prostate cancer, AR still retains its function contributing to disease progression. Here we demonstrate that emodin, a natural compound, can directly target AR to suppress prostate cancer cell growth *in vitro* and prolong the survival of C3(1)/SV40 transgenic mice *in vivo*. Emodin treatment resulted in repressing androgen-dependent transactivation of AR by inhibiting AR nuclear translocation. Emodin decreased the association of AR and heat shock protein 90 and increased the association of AR and MDM2, which in turn, induces AR degradation in a ligand independent manner. Thus, emodin, through targeting AR may serve as a novel therapeutic and preventive agent for prostate cancers.

Significance

Hormone refractory relapse is an inevitable and lethal event for advanced prostate cancer patients after hormone deprivation. A recent prostate cancer prevention trial and a growing body of evidence indicate that hormone deprivation may promote this aggressive prostate cancer phenotype. Notably, androgen receptor is the key player for the relapse transition. This provides a strong rationale for searching new effective agents targeting the downregulation of androgen receptor to treat advanced prostate cancer. We report here that emodin, a natural compound can efficiently downregulate androgen receptor through a proteasome-mediated pathway and inhibit prostate cancer cells growth in vitro and in vivo. Our work indicates a new mechanism for the emodin-mediated anticancer effect and justifies further investigation of emodin as a therapeutic and preventive agent for prostate cancer.

Introduction

Prostate cancer (PCa) is the most common malignant disease and the second leading cause of death in U.S. male cancer patients. Despite that diagnosis is earlier than in the past, the incidence and mortality rates of this cancer are still increasing steadily. Approximately 189,000 cases diagnosed and 30,200 deaths were attributed to the disease in 2002 (Jemal et al., 2002), and inevitably, 28,900 men are expected to die of this disease in 2003 (Cancer facts & figures). Upon this devastating disease with tremendous impact on public health, unfortunately, the effective treatment options are limited and metastatic disease frequently develops even after potentially curative surgery or radiation therapy (Petrylak, 1999; Pisters, 1999; Richie, 1999). Besides focusing on early diagnosis and treatment of this long-term and multiple-step malignant disease, prevention may be an alternative and more effective approach.

A recent and exciting prevention trial for PCa has been done to show that finasteride, a 5-α reducatase inhibitor which inhibits the conversion of testosterone to a more potent androgen - dihydrotestosterone, has the chemopreventive effect for PCa development (Thompson et al., 2003). Their results support that prevention could be a right direction and strategy while dealing with PCa, but this study points out that finasteride also increases the risk of high-grade PCa. One possible explanation for the

outcome may have resulted from the fact that finasteride reduced the intraprostatic dihydroteststerone level which created an environment more beneficial for those less androgen dependent high-grade cancers to grow (Morgentaler et al., 1996; Scardino, 2003). Consistent to this notion, previous reports showed that men who developed prostate cancers with low testosterone levels have higher Gleason grades and worse outcomes than those with normal testosterone levels (Ishikawa et al., 1989; Prehn, 1999; Schatzl et al., 2003). The risk of death within 15 years due to high grade PCa that are managed conservatively ranges from 42 percent to 87 percent, much higher than the low grade PCa ranges from 4 to 30 percent (Albertsen et al., 1998). Thus, although the clinical trial was successful, the results do not provide a clear resolution for patients and physicians to choose finasteride as a preventive agent due to the potential high risk for development of high grade PCa which is associated with much higher mortality rate.

The PCa depends on androgen receptor (AR) to mediate androgen's effect on tumor initiation and progression (Chang et al., 1995). The standard hormone therapy for PCa aims at inactivating AR transcriptional activity by means of androgen deprivation (through surgical or medical castration) or androgen blockade (with AR antagonists) (Forster et al., 2002; Huggins, 1941). The same concept has been applied to PCa prevention. However, for those advanced PCa, this response is temporary; as

disease progresses, almost all PCas eventually become androgen independent. More and more evidence suggest that hormone therapies for prostate cancer may promote the phenotypic progression of those tumor cells that are able to survive the acute period of the therapy (Culig et al., 1999; Kokontis et al., 1998). Although PCa uses various schemes to subvert normal restraints on cell growth along with deprivation of androgen, a common feature among the diverse schemes is that the AR is still expressed and required for androgen-independent PCa cell growth (Arnold and Isaacs, 2002; Feldman and Feldman, 2001; Grossmann et al., 2001; Zegarra-Moro et al., 2002). These tumors are androgen independent, but they appear to remain AR dependent. Thus, it is important to develop new compounds that can inhibit AR function in an alternative, ligand-independent manner.

In the present study, we found that emodin (1,2,8-trihydroxy-6-methylanthraquinone), a natural compound extracted from *Rheum palmatum,* inhibits AR transcriptional activity by preventing AR nuclear translocation. Emodin treatment results in decreasing the association of AR and hsp90 but increasing the association of AR and MDM2, in turn inducing AR degradation in a ligand independent manner. Most important, we showed that through targeting AR, emodin can suppress PCa cell growth *in vitro* and prolong the survival of PCa-producing C3(1)/SV40 transgenic mice *in vivo*. These results indicate a new

mechanism for the emodin-mediated anticancer effect and justify further investigation of emodin, a natural compound as a therapeutic and preventive agent for PCa.

Results

Emodin Inhibits Cell Proliferation of AR-positive Prostate Cancer cells. As an initial attempt to test emodin's effect on PCa cells, we treated AR-positive LNCaP cells with various concentrations of emodin in the presence or absence of synthetic androgen R1881. As expected, the AR-positive LNCaP cells response and proliferate upon synthetic androgen R1881 stimulation (Horoszewicz et al., 1983). Emodin efficiently inhibited LNCaP cell proliferation stimulated by R1881 in a dose-dependent manner within 72 hours (Fig. 1A). To further investigate emodin's antiproliferative effect, we treated another prostate cancer cell line, DU-145 with various concentrations of emodin. DU-145 cells, which are derived from a brain metastasis, do not express the AR and grow independent of androgen. The AR-negative DU-145 cells are more resistant to the emodin-mediated antiproliferative effect than the AR-positive LNCaP cells (Fig. 1B). Although AR is not the only difference between LNCaP and DU-145 PCa cells, this result raises an interesting possibility that AR-positive PCa cells may be more sensitive to emodin treatment. To extend this observation, we tested emodin's effect on a pair of AR-negative and AR-positive PCa cell lines, PC3 and PC3-AR. PC3 is a well-defined AR-negative PCa cell line and PC3-AR is a clonal PC3 cell line stably transfected with androgen

receptor. Thus, they have identical genetic background except for the AR status. Again, the AR-positive PC3-AR cells are much more sensitive to emodin treatment than the AR-negative PC3 cells (Fig. 1C). Treatment with low dose (10 μM) emodin yielded a significant inhibition of PC3-AR cell growth by 35 % within 72 hours, compared with parental PC3 cell growth by 7 % ($P < 0.01$), high dose (40 μM) emodin showed some inhibition of PC3 cell growth by 22 % but more significant inhibition of PC3-AR cell growth by 60 % ($P < 0.001$). The antiproliferative effect of the PC3-AR cells was evident already 24 hours after emodin treatment but not of PC3 cells (data not shown). Thus, this result is consistent with the results showed in Fig. 1B and supports the notion that AR-positive PCa cells are more sensitive to emodin treatment.

Emodin Inhibits AR Transcriptional Activity. Since AR mediates androgen's effect on cell proliferation and survival in LNCaP cells, and AR-positive PCa cells are more sensitive to emodin treatment, we hypothesize that emodin inhibits AR's function. To test this hypothesis, we investigated emodin's effect on AR downstream target gene expression. RT-PCR and western blot analysis showed that expression of prostate specific antigen (PSA), an AR-targeting gene was downregulated by emodin (Fig. 2A & 2B). To further test emdon's effect on AR transcriptional activity, we transiently transfected LNCaP cells with PSA-Luciferase and probasin-Luciferase reporters, two

well-characterized AR targeting promoters. Both androgen-mediated PSA and probasin promoter activities were repressed in emodin-treated cells in a dose-dependent manner (Fig. 2C). The emodin-mediated repression of PSA and probasin reporter activities were also observed in PC3 cells with the cotransfection of AR-expressing vector, pSG5AR plasmid and reporters (Fig. 2D). Without cotransfection of pSG5AR, these two reporters will not respond to R1881 stimulation and emodin has no effect on their promoter activities in the AR-negative PC3 cells (data not shown). Thus, emodin inhibits the transcriptional activity of AR.

Emodin Inhibits AR Nuclear Translocation. The function of AR is closely related to its subcellular localization. Because emodin can inhibit AR transcriptional activity, we examined emodin's effect on AR subcellular localization. The intracellular distribution of AR in LNCaP cells was assessed using immunofluorescence microscopy. AR was mostly localized in cytosol in the absence of androgen for 24 h (Fig. 3A). After stimulation with 0.1 nM R1881 for 2 h, AR translocated into the nucleus as clearly indicated by the yellow staining in the nucleus merged from AR (green) and nuclear DAPI (red) staining. However, when cells were treated with both R1881 and emodin, AR was mostly retained in the cytosol as shown by the AR staining (green) in the cytosol and reduced yellow staining in the nucleus. The emodin-mediated cytosolic retention of AR was also supported by the biochemical

approach from cellular fraction experiments (Fig. 3B). To further investigate the specific effect of emodin on AR nuclear localization, we performed the time lapse experiment by using a fusion protein between AR and GFP to dynamically evaluate AR trafficking in a single living cell. We transfected the AR-GFP fusion protein to COS-1 cells, and then tested the effects of different kinds of inhibitors - emodin, AG1478 (a tyrosine kinase inhibitor), genistein (a tyrosine kinase inhibitor), Ly294002 (a PI3 kinase inhibitor), and U0126 (a MAP kinase inhibitor) - on AR nuclear localization under different time periods. Before proceeding this experiment, all the inhibitors were tested and titrated the optimal dosage for inhibition of their functional targets (data not shown). Under the stimulation of R1881 for two hours, only emodin but not the rest inhibitors prevented AR nuclear translocation (Fig.3D). This effect can be observed for up to 24 h (data not shown).

Emodin induces androgen receptor degradation. Since androgen stimulation induces AR nuclear translocation and emodin inhibits this activity resulting in AR accumulation in the cytosol, it becomes very interesting to further investigate the fate of AR under this condition. To this end, we treated LNCaP cells with various concentrations of emodin or for different lengths of time in the presence or absence of 0.1 nM R1881 and measured AR protein expression. A time- and dose-dependent reduction of the AR protein level was observed (Fig. 4A, 4B). Although AR is more

stable and has a higher basal level of expression in the presence of ligand, emodin can efficiently deplete AR even in cells undergoing synthetic androgen R1881 stimulation. Thus, emodin-induced depletion of AR is ligand-independent. To investigate the molecular mechanisms for AR depletion, we first investigate whether emdoin may have effect on AR mRNA expression. We analyzed AR mRNA level under the treatment with various concentrations of emodin by RT-PCR and found that emodin did not influence AR mRNA level (Fig. 4C). We then further test emodin's effect on AR protein stability. By using cyclohexamide to inhibit protein synthesis, the AR protein stability was significantly reduced under emodin treatment (Fig. 4D). To test whether emdoin induce AR degradation through the proteasome pathway, treatment with the proteasome inhibitor MG132 resulted in a marked suppression of emodin-induced AR depletion (Fig. 4E). These phenomenon were also observed in PC3-AR cells (Fig. 4F). Taken together, these findings indicate that emodin induces AR degradation through a proteasome-mediated pathway.

Emodin disrupts AR-hsp90 association and increases AR-MDM2 association and ubiquitination. AR is known to form a heteromeric complex with two molecules of hsp90 which has been shown to participate in regulating the protein stability of ligand-unbound AR. Previous reports showed that some tyrosine kinase inhibitors can reduce the AR protein level (Fritz et al., 2002). We further tested the efficacy of

emodin and other tyrosine kinase inhibitors such as genistein and AG1478 on the reduction of AR and the interaction between AR and hsp90. Due to different drugs have different kinetic and dynamic activities, we chose a relative high dose of AG1478 (10 μM) and genistein (50 μM) to ensure their efficacy (Gao et al., 2004; Steinbach et al., 2004). Under this situation, emodin-induced reduction of AR is more potent than the other two tyrosine kinase inhibitors especially in the presence of R1881 (Fig. 5A). In addition, only emodin can significantly disrupt the association between AR and hsp90, whether treated for a short (2 h) (Fig. 5B) or a longer period (8 h) (data not shown). This result indicates that emodin induces dissociation of AR and hsp90 involving a novel mechanism different from other tyrosine kinase inhibitors. The emodin-induced dissociation between AR and hsp90 may render the incomplete AR heterocomplex more vulnerable to degradation through the proteasome-mediated pathway. It has been shown that AR can be ubiquitinated by an E3 ligase, MDM2 (Forster et al., 2002).We hypothesized that dissociation of AR and hsp90 by emodin increases the interaction between AR and the E3 ligase MDM2 and therefore enhances degradation. To test this hypothesis, we measured the interaction between AR and MDM2 with or without emodin treatment in LNCaP cells. Emodin treatment increased the association between the endogenous MDM2 and AR as evident from co-immunoprecipitation experiments using antibodies against MDM2

and AR (Fig. 5C). The emodin-induced AR-MDM2 association was also demonstrated in 293 cell line through cotransfection of AR and MDM2 expression vectors (data not shown) suggesting this is a general phenomenon in different cell types. In addition, emodin treatment resulted in an increase of AR ubiquitination (Fig. 5D). Taken together, these results indicate that emodin dissociates hsp90 from AR, and enhances AR and MDM2 association, which may lead to further ubiquitination and degradation.

Emodin downregulates AR and prolongs survival of C3(1)/SV40 transgenic mice. The above results established a novel molecular mechanism to explain how emdoin may downregulate AR in *in vitro* cell culture system. To further investigate whether emodin also has the same effect in *in vivo* animal models, we chose C3(1)/SV40 transgenic mice as an experimental model. It is known that the male mice of this strain will develop AR-positive PCa and eventually die with PCa because the SV40 large T antigen, an oncogene was driven by the promoter of rat prostatic steroid binding protein [C3(1)] gene in the transgenic mice. Since the carcinogenesis of C3(1)/SV40 transgenic mouse model is primarily driven by AR, this transgenic mouse model provides a clean background and by using the tumor development as a readout, we can more specifically test emodin's effect on AR *in vivo*. We treated male C3(1)/SV40 transgenic mice with either emodin (40 mg/kg) or DMSO

intraperitoneally every other day while the mice were four weeks old and no signs of tumors. Emodin-treated mice have significantly longer survival than the control group ($P < 0.001$) (Fig. 6A). Emodin-treated mice maintained the body weight gain, in contrast, DMSO-treated control mice significantly lost body weight gain after the age of 20 weeks ($P < 0.05$) (Fig. 6B). We noticed that not only the size but also the hair grooming and cage activity were clearly different between emodin-treated and control groups. In general, emodin-treated mice looked much healthier but the control mice appeared to be in distress with labored breathing, cachectic and lethargic patterns (Fig. 6C). This result showed that emodin not only has low drug toxicity but also maintains the physical activity of C3(1)/SV-40 transgenic mice by preventing tumor progression. To further address whether the biological effect of emodin is related to its activity to downregulate AR, we analyzed AR expression of tumor tissues from both emodin-treated and control groups. Immunohistochemical staining using an AR-specific antibody clearly indicated that PCa tumor tissues from emodin-treated mice were much weaker than those from the control group (Fig. 6D, 6E). The same results were also obtained by using western blot analysis of fresh tumor samples taken from other pairs of mice (Fig. 6F). In addition, when we analyzed the tumor progressive status by histopathological investigation of mice at 21 weeks of age, the emodin-treated mice clearly had a lower incidence of tumor invasion to the

periurethral muscle structure (1/7) compared to the control group (7/7) (Fig. 6G). Our results showed that emodin, indeed can downregulate AR in C3(1)/SV-40 transgenic mice model. Considering the success of the recent finasteride prevention trial for PCa and the dilemma it created due to its androgen deprivation nature, emodin, which directly downregulates AR in a ligand-independent manner, may have an advantage for further development as a therapeutic and chemopreventive agent for PCa.

Discussion

Hormone refractory relapse is an inevitable and lethal event for advanced prostate cancer patients after hormone deprivation. Extensive studies have been devoted to defining the molecular mechanisms required for the refractory state. A growing body of evidence points out that AR is not only responsible for PCa initiation but also a key player of hormone refractory transition. These observations provide a strong rationale for aiming AR as both a therapeutic and preventive target for PCa. Because AR is a transcriptional factor and mediates its activity through genotropic mechanisms, it is plausible to envision ideal drugs that prevent AR nuclear translocation or downregulate AR levels to impair its effects on target genes for tumor development and disease progression. Our present work unexpectedly found out a natural compound, emodin may have the potential to fulfill this requirement.

Emodin (1,3,8-trihydroxy-6-methylanthrauinone), an active extract of *Rheum palmatum*, has been shown to have multiple biological activities, including anti-inflammatory, antibacterial, diuretic, immunosuppressive, vasorelaxant, and anticancer effects (Kuo et al., 2001; Lee, 2001; Sato et al., 2000; Zhang et al., 1999). In addition, we found a novel emodin-mediated mechanism of inhibition of PCa cell

growth, especially AR-positive cells, involving sequential events effect on inhibition of AR transcriptional activity and downregulation of AR expression.

AR is a nuclear transcription factor. Nuclear translocation is a key step for AR in response to androgen stimulation (Zhou et al., 1994), and this process initiates the transactivation of downstream target genes to promote PCa cell proliferation and survival. Our results showed that emodin inhibits androgen-mediated AR nuclear translocation and also induces dissociation of AR and hsp90. However, this phenomenon did not occur in the treatment with other kinase inhibitors involving receptor tyrosine kinase, MAP kinase and Akt pathways. In consistent to the previous reports, the association between AR and hsp90 is not disrupted by other tyrosine kinase inhibitor (Bagatell et al., 2001). These results suggest that emodin-mediated inhibition of AR nuclear translocation is specific and involves a novel mechanism correlating with the dissociation of AR and hsp90. It has been shown that hsp90 function is required for AR nuclear translocation which is consistent with our observation (Georget et al., 2002).

The steroid receptors interact with hsp90 and other cochaperones to create a mature conformation for proper protein function (Fang et al., 1996; Georget et al., 2002; Segnitz and Gehring, 1997). Without hsp90 binding, the misfolded or unfolded proteins will be recognized and degraded by the ubiquitin-proteasome system (Pickart,

2001). Emodin treatment induces the dissociation between AR and hsp90 and increases the association of AR and MDM2, providing a plausible mechanism for the involvement of MDM2 as an E3 ligase for the emodin-meidated AR degradation. Previous studies have shown that the hsp90 inhibitors, such as geldanamycin and its derivatives, can directly bind to the ATP-binding pocket of hsp90 and inhibit its function then further induces steroid receptor degradation (Bagatell et al., 2001; Lee et al., 2002; Segnitz and Gehring, 1997; Solit et al., 2002; Vanaja et al., 2002). Although both emodin and geldanamycin share the similar feature of abrogating the interaction between AR and hsp90, their mechanisms are different. Emodin induces dissociation of AR and hsp90, but geldanamycin cannot. It will be interesting to see whether MDM2 is also responsible for the geldanamycin-induced AR degradation.

A recent study showed that overexpression of AR in hormone refractory xenograft model is consistent with observations in human clinical specimens and overexpression of AR promote hormone dependent xenograft into hormone independent (Chen et al., 2004; Linja et al., 2001). These observations indicate that reducing AR expression to a critical level would contribute to preventing PCa progression. Emodin-induced degradation of AR occurs in a ligand-independent manner. Thus, as long as AR is functional in PCa regardless androgen dependent or independent, emodin should inhibit cancer cell growth because of the induction of AR

degradation. In addition to enhanced AR expression, other proposed mechanisms include cross-talk between AR and other signal transduction pathways also involved in development of the hormone refractory state. Overexpression of receptor tyrosine kinases such as HER2/*neu* also is known to contribute to PCa development (Signoretti et al., 2000). In this regard, it should be mentioned that high dose emodin also associates with the activity to inhibit tyrosine kinase and suppress HER2/*neu*-mediated tumorigenecity in breast cancer cells (Zhang et al., 1995; Zhang et al., 1999). The dual functions of anti-AR and anti-tyrosine kinase may be an advantage for using emodin as a chemopreventive agent to avoid the development of aggressive PCa phenotype.

In addition to the observation of emodin's effects in the *in vitro* cell culture system, the *in vivo* effects were also observed in emodin-treated C3(1)/SV40 transgenic mice. Our results showed that emodin induced degradation of AR in the tumor tissues, suppressed tumor development and prolong animal survival. Since the carcinogenesis of C3(1)/SV40 transgenic mice model is primarily driven by AR, emodin-induced degradation of AR contributes to its chemopreventive effect on inhibition of tumor development in C3(1)/SV40 transgenic mice. Emodin-treated mice maintained their body weight gain and physical activity suggesting that the effective dose of emodin which suppresses tumor progression is well-tolerable and

nontoxic. Tumor invasion resulting in distant metastases is the major cause of PCa-related death (Waples, 1999). Emodin-treated transgenic mice had lower incidence of periurethral invasion which represents the preventive effect of emodin contributing to prolong the survival of transgenic mice.

Considering the dilemma created by the recent Prostate Cancer Prevention Trial (PCPT), this current study provides the evidence to support that direct targeting AR rather than its ligands could be a good strategy in the treatment or prevention of PCa. Emodin may have the potential as a novel anti-AR therapeutic and preventive agent for PCa.

Experimental procedures

Cell Culture. LNCaP, PC3, DU145, 293 and COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in serum-containing media as recommended by the supplier. PC3-AR cells are a clonal cell line derived by stable transfection of PC3 cells with a plasmid containing the coding region of the human androgen receptor. PC3-neo cells were stably transfected with the same vector lacking the AR cDNA sequence. Medium for PC3-AR cells also contained the selective antibiotic G418 (GIBCO/BRL, Gaithersburg, MD), 400 μg/ml. For experiments requiring an androgen-depleted condition, cells were incubated in phenol red-free RPMI 1640 medium supplemented with 5% charcoal-stripped fetal bovine serum (c-FBS) for 1 day before initiation of the experiment.

Reagents and Plasmids. The synthetic androgen R1881 (PerkinElmer, Boston, MA) was dissolved in 100% ethanol and stored at -20°C for up to 1 month. Emodin (Sigma Co., St. Louis, MO), MG132, AG1478, LY294002, U0126, and genistein (Calbiochem, San Diego, CA) were dissolved in dimethyl sulfonide (DMSO). Anti-AR antibody (15061, 15071), anti-MDM2 antibody, and anti-hsp 90 antibody were purchased from PharMigen (San Diego, CA), Oncogene (San Diego, CA) and

Santa Crutz Co. (Santa Cruz, CA) respectively. Expression plasmids used were pSG5-AR, pcDNA3-MDM2, and pcDNA3.1-GFP-AR. The pSG5-AR was generated by inserting the human AR cDNA into the EcoRI and BamHI site near the start and termination codons of expression vector pSG5. The pcDNA3-MDM2 was generated as previously described (Zhou et al., 2001). The pcDNA3.1-GFP-AR was kindly provided by Dr. Zhengxin Wang (The University of Texas, M.D. Anderson Cancer Center, Houston, TX).

Western Blot Analysis and Immunoprecipitation. LNCaP cells were treated with emodin at different concentrations for 18 h. The cells were subjected to lysis with RIPA buffer (20 mM Na_2PO_4, pH 7.4; 150 mM NaCl; 1% Triton X-100; 1% aprotinin; 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 mg/ml leupeptin; 100 mM NaF; and 2 mM Na_3VO_4). Protein levels were quantitated using the Lowry method (Bio-Rad Laboratories). Approximately 100 μg of protein per sample was resolved by 8% sodium dodecylsulfae-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. Membranes were placed in a blocking solution of 5% non-fat milk (1x PBS, 0.05% Tween 20) for 30 min at room temperature and then incubated with anti-AR monoclonal antibody 15061 at a dilution of 1:500 for 1 hour at room temperature or overnight in a cold room. After three 10-min washes, the membrane was then incubated with 5% milk buffer with HRP

goat anti-mouse antibody at a dilution of 1:5000 for 40 min at room temperature. The membrane was washed as described and reacted for 1 min with chemiluminescent reagents commercially supplied (ECL). For the time course responsiveness, LNCaP cells were treated with emodin (40 μM) for different time intervals. Then the androgen receptor protein levels were examined by immunoblotting analysis as described above. To check the effect of proteasome inhibitor on emodin-induced depletion of androgen receptor, LNCaP cells were treated with emodin (40 μM) and the proteasome inhibitor simultaneously for 6 hours. The controls were treated with the vehicle (DMSO) alone. The preparation of total protein lysates and subsequent immunoblotting analyses were performed as described above. The intensity of the protein signal was quantitated by BIO-RAD PDQUEST Image software. The results were calculated as the percentage of the controls and normalized with β-actin.

RT-PCR. Total RNA was extracted from cells and RT-PCR was done as per the manufacturer's instructions (SuperScript Preamplificaton system; Life Technologies, Inc.). The primers derived from the AR coding sequence (5'-CTCACCAAGCTCCTGGACTC-3') and PSA coding sequence (5'-GCAGCATTGAACCAGAGGAG-3') were used to amplify the AR and PSA transcripts. Primers for GAPDH Internal control were: 5'-AGGTGAAGGTCGGAGTCAAC-3' and 5'-TCCATTGATGACAAGCTTCCC-3'.

Amplification was done on a Perkin Elmer DNA cycler 480 for 35 cycles with denaturing at 94 °C for 30 s, annealing at 58 °C for 1.5 min, and extension at 72 °C for 1.5 min.

Transient transfection and luciferase assay. LNCaP and PC3 cells were plated 1 day before transfection at a density of 2 x 10^5 cells/well in six-well plates. The LNCaP cells were cotransfected with a luciferase reporter plasmid (0.3 μg of PSA-luc or Probasin-luc) and a β-galactosidase expression plasmid (0.2 μg of CMV-β-gal) and expression plasmids with an empty vector (0.9 μg each) as indicated using liposomes. For PC3 cells, the procedure was the same, except that these cells were cotransfected with additional AR plasmid (0.3 μg of pSG5-AR). After transfection, the cells were cultured in phenol red-free medium supplemented with 5% c-FBS in the absence or presence of the synthetic androgen R1881 (PerkinElmer; 0.1 nM) and various doses of emodin. Cell lysates were collected 48 h after transfection, and the luciferase activity of each sample was measured using a luciferase assay kit (Promega). β-galactosidase activity was determined to normalize variations in transfection efficiency.

[3H]-Thymidine incorporation assay and FACS analysis. Cells were detached by trypsinization. 2000 cell were plated on a 96-well microtiter plate overnight. Then the medium was replaced with phenol red free RPMI medium supplemented with 5%

c-FBS overnight. The cells were then treated with different concentration of emodin (0, 10, 20 and 40 μM) with or without simultaneous stimulation of R1881 (0.1 nM) for 20 hours. For the control, DMSO and ethanol were added. Six hours before completion of the experiment, 1 μCi of thymidine was added to each well. The cells were then harvested and radioactivity was measured. For the apoptosis assay, cells grown on 60 mm tissue culture dishes to 70% confluency were trypsinized, pelleted, washed with chilled PBS, and resuspended in 500 μl PBS. 5 ml ice-cold ethanol was added to the cell suspension and vortexed. The cells were incubated at -20°C overnight. Fixed cells were washed twice with PBS. 30 min prior to proceeding the flow cytometry analysis, 300 ul working solution (0.1% sodium citrate, 0.1% Triton-X, 8-20 μg RNAse/ml, propidium iodide 50 μg/ml) was added. The DNA content of the cells was analyzed with cell quest software on a Becton-Dickinson flow cytometer.

Fluorescence Imaging and Fractionation. For immunodetection, cells were cultured in four chambered glass slides and treated with or without 40 uM emodin in the presence of 0.1 nM R1881 then fixed with 100% methanol at -20°C for 10 min. Fixed cells were rinsed twice with PBS (10 min each wash) and air dried. Before antibody treatment, slides were incubated in a humidified chamber for 30 min, followed by overnight incubation (at 4°C) with FITC-conjugated polyclonal anti-AR antibody (diluted 1:100). After extensive washing with PBS, the samples were examined under

a fluorescent microscope (Zeiss). Fluorescence imaging of living cells were performed through an Axiovert 200 inverted fluorescence microscope. COS-1 cells were transiently transfected with chimeric GFP plasmid (pcDNA3.1-GFP-AR) and allowed to express chimeric protein for 24 h. The cells were first observed without any treatment and then with addition of different inhibitors for 30 min followed by addition of 0.1 nM R1881 into the same chamber. The same living cell was studied and recorded at different time periods. For cell fractionation, briefly, freshly harvested cells were suspended in 5 x volume of a lysis buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM $MgCl_2$, 0.5% Nonidet P40, 1 mM Na_3VO_4, 1 mM PMSF, 0.15 U ml^{-1} aprotinin) and homogenized by 30 strokes in a tightly fitting Dounce homogenizer. The homogenate was centrifuged at 1,500 *g* for 5 min to sediment the nuclei. The supernatant was then resedimented at 15,000 *g* for 5 min, and the resulting supernatant formed the non-nuclear fraction. The nuclear pellet was washed three times and resuspended in the same buffer containing 0.5 M NaCl to extract nuclear proteins. The extracted material was sedimented at 15,000 *g* for 10 min and the resulting supernatant was termed the nuclear fraction.

In vivo transgenic mice study. Three pairs of C57BL/6J-TgN C3(1)/SV40 Tag-transgenic mice were purchased from Jackson Laboratory (Bar Harbor Maine) and the transgenic progeny were identified by PCR analysis of tail DNA isolated from

3-week-old litters using standard techniques. We treated four-week-old mice with DMSO or emodin (40 mg per kg per d) by intraperitoneal (i.p.) injection. Each treated mouse received supplemental dosages at 140 days intervals and the efficacy of the treatment was measured by body weight and survival. Additional groups of mice (6-7 mice/group) were given treatment as described above but sacrificed at 21 weeks of age for histological and western blot analysis.

Immunohistochemical staining. Immunohistochemical analysis for AR protein expression in tumor samples was done as described (Xing et al., 2000). Polyclonal antibody against AR was purchased from Upstate (Waltham, MA).

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Figure legends

Fig. 1. Effect of emodin on prostate cancer cells proliferation. **A**, LNCaP cells were treated with DMSO and various concentrations (10 μM , 20 μM, 40 μM) of emodin, respectively, in the presence or absence of 0.1 nM R1881 for 72 h. The percentage of viable cells after treatment comparing to control (DMSO treated defined as 100%) were determined by MTT assay. **B**, LNCaP and DU-145 cells were treated with DMSO or 40 μM emodin for 24, 48, and 72 h, respectively. The percentage of viable cells after treatment comparing to control were determined by MTT assay. **C**, PC3, PC3-AR cells were treated with DMSO, 10 μM or 40 μM emodin for 72 h. The percentage of viable cells after treatment was determined by MTT assay and compared to control.

Fig. 2. Effects of emodin on AR transcriptional activity. **A**, LNCaP cells were treated with solvent (DMSO, labeled D) and various concentrations of emodin (10-40

μM, labeled E10-E40) for 18 h with or without (labeled C) 0.1nM R1881.The expression of AR-target gene PSA was analyzed by RT-PCR. The expression of GADPH was monitored as a control. **B**, The PSA protein level was analyzed by western blotting after emodin treatment and tubulin was measured to ensure consistent loading. **C**, PSA and probasin luciferse reporter genes was transiently transfected into LNCaP cells and the luciferase activity was measured after emodin treatment. **D**, Same as in **C** except PC3 cells was co-transfected with pSG5AR plasmid and reporters. The corresponding β-galactosidase activity was used to normalize luciferase activity. Data represent the mean ± SD of three independent experiments.

Fig. 3. Effect of emodin on AR nuclear translocation. **A**, LNCaP cells were treated with or without 40 μM emodin for 30 min, then treated with or without 0.1 nM R1881 for an additional 2 h. Cells were fixed in cold methanol and immunostained with FITC-conjugated anti-AR antibody. **B**, Results of fractionation experiments on LNCaP cells treated as described in **A**. The AR in the cytosol (C) and nuclear extracts (N) was detected by anti-AR antibody. Anti-PARP and anti-tubulin antibodies were run to distinguish between the nuclear and cytosolic fractions, respectively. **C**, COS-1 cells were transiently transfected with pGFP-AR and treated with emodin (40 μM), AG1478 (10 μM), genistein (50 μM), Ly294002 (20 μM), or U0126 (20 μM) in the

presence of 0.1 nM R1881 for 2 h. The GFP-AR fusion proteins were detected in living cells using an Axiovert 200 inverted fluorescence microscope. The GFP-AR, DAPI, and merging of these two signals are represented as *green*, *red*, and *yellow*, respectively.

Fig. 4. Effect of emodin on AR expression. A, LNCaP cells were treated with solvent (DMSO, labeled D) and various concentrations of emodin 5,10, 20, or 40 μM (labeled E5-E40) for 18 h with or without 0.1 nM R1881. The AR protein level was analyzed by Western blot and quantitated by Bio-Rad PDQUEST Image software and plotted as the percentage of the control (without emodin) after normalization with actin. **B**, LNCaP cells were treated with 40 μM emodin for various lengths of time. The AR protein level was measured by immunoblotting. **C**, The expression of the AR gene in LNCaP cells was analyzed by RT-PCR after treatment with various concentrations of emodin for 18 h. The expression of GADPH was monitored as a control. **D**, LNCaP cells were treated with 40 μM emodin and 10 μM cyclohexamide for various length of time. DMSO was added to the control. The AR protein level was measured by Western blot analysis. **E**, LNCaP cells were treated with 40 μM emodin and 5 μM MG-132 with or without R1881 for 12 h. DMSO was added to the control. The AR protein level was measured by Western blot analysis. **F**, PC3-AR cells were treated with 40 μM emodin and 5 μM MG-132 for 12 h. DMSO was added to the

control. The AR protein level was measured by Western blot analysis.

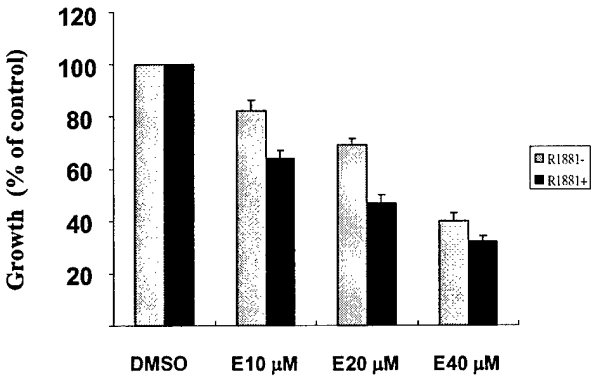
Fig. 5. Effect of emodin on the associations between AR and hsp90 and AR and MDM2. A, LNCaP cells were treated with emodin (40 μM), AG1478 (10 μM), and genistein (50 μM) with or without R1881 for 18 h. The AR protein level was measured by Western blot analysis. **B**, LNCaP cells were incubated with c-FBS medium overnight then treated with or without emodin (40 μM), AG1478 (10 μM) and genistein (50 μM) for 2 h. Immunoprecipitation and western blot analysis were performed with indicated antibodies. **C**, LNCaP cells were incubated in c-FBS medium in the presence or absence of 0.1 nM R1881 overnight, then treated with or without emodin (40 μM) in the presence of MG-132 (10 μM) for 2 h. AR immunoprecipitation was done as in Fig. 2C except that immunoblotting was performed with anti-MDM2 antibody rather than anti-hsp90 antibody. **D**, LNCaP cells were incubated in c-FBS medium overnight, then treated with or without emodin (40 μM) in the presence of MG-132 (5 μM) and with or without 0.1 nM R1881 stimulation for 8 h. AR immunoprecipitation was done as in Fig. 2B except that immunoblotting was performed with anti-ubiquitin antibody rather than anti-hsp90 antibody.

Fig. 6. Effect of emodin on C3(1)/SV40 transgenic mice. A, Four-week-old mice were i.p. injected with 40 mg/kg emodin or DMSO every other day. Survival of mice

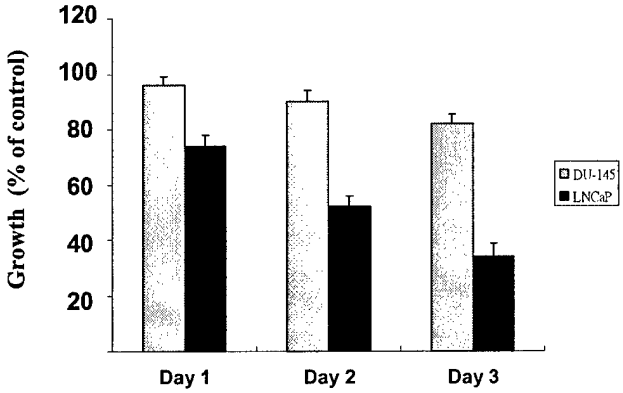
treated with emodin or DMSO are shown. **, $P < 0.001$. **B**, The body weight gain profiles of emodin-treated and control transgenic mice from 0-30 weeks are shown. *, $P < 0.05$. **C**, The emodin-treated mouse maintained body weight gain and physical activity not seen in the control mouse (24-week-old). **D**, Tumor tissue sections from 21-week-old emodin-treated and control mice with identical treatment as survival experiment shown in **a**, were stained with antibody specific to AR. **E**, Quantitative analysis of AR expression between emodin-treated and control mice were assessed in 2500 cells in several different views ($P < 0.05$). **F**, Western blot analysis of fresh tumor tissues obtained from two sets of 21-week-old emodin-treated and control mice with identical treatment as survival experiment shown in **A**. **G**, H&E-stained tumor tissue sections from the emodin-treated and control mice are shown (100 x, low magnification; 400 x, high magnification). The control mice showed tumor invasion of periurethral muscle (black arrow) not seen in emodin-treated mice.

Figure 1

A


120
100
80
60
40
20
Growth (% of control)
R1881-
R1881+
DMSO
E10 μM
E20 μM
E40 μM

B


120
100
80
60
40
20
Growth (% of control)
DU-145
LNCaP
Day 1
Day 2
Day 3

C

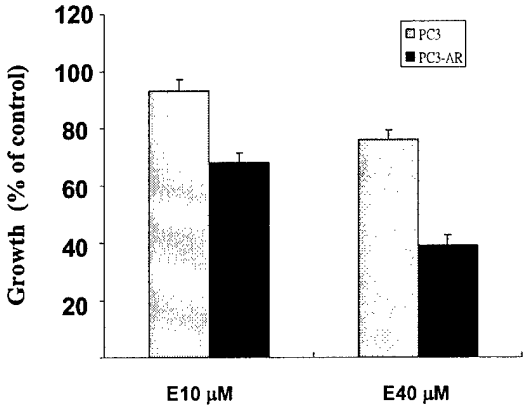

120
100
80
60
40
20
Growth (% of control)
PC3
PC3-AR
E10 μM
E40 μM

Figure 2

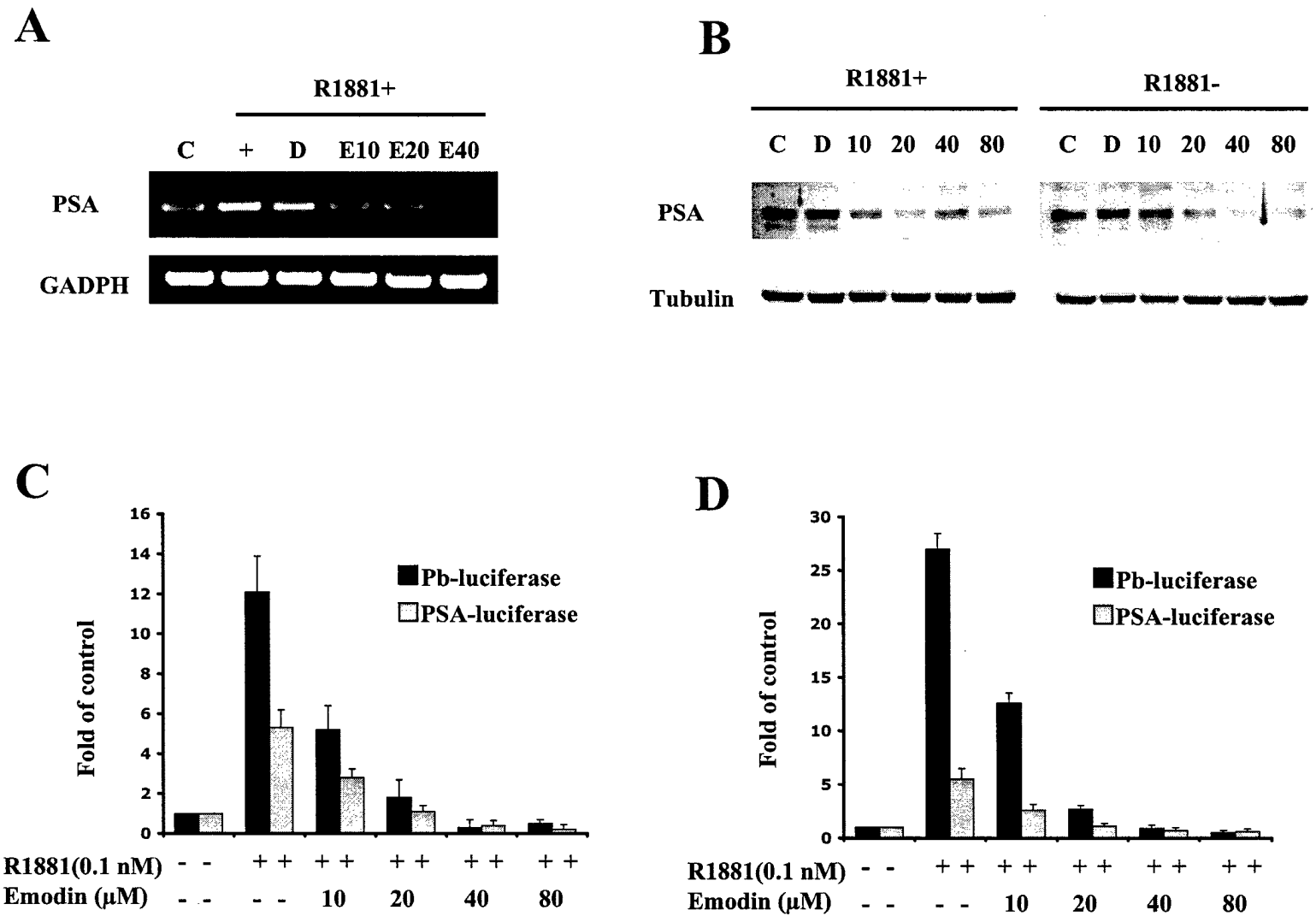

A
R1881+
C + D E10 E20 E40
PSA
GADPH
B
R1881+
R1881-
C D 10 20 40 80 C D 10 20 40 80
PSA
Tubulin
C
Fold of control
Pb-luciferase
PSA-luciferase
R1881(0.1 nM) - - + + + + + + + + + +
Emodin (μM) - - - - 10 20 40 80
D
Fold of control
Pb-luciferase
PSA-luciferase
R1881(0.1 nM) - - + + + + + + + + + +
Emodin (μM) - - - - 10 20 40 80

Figure 3

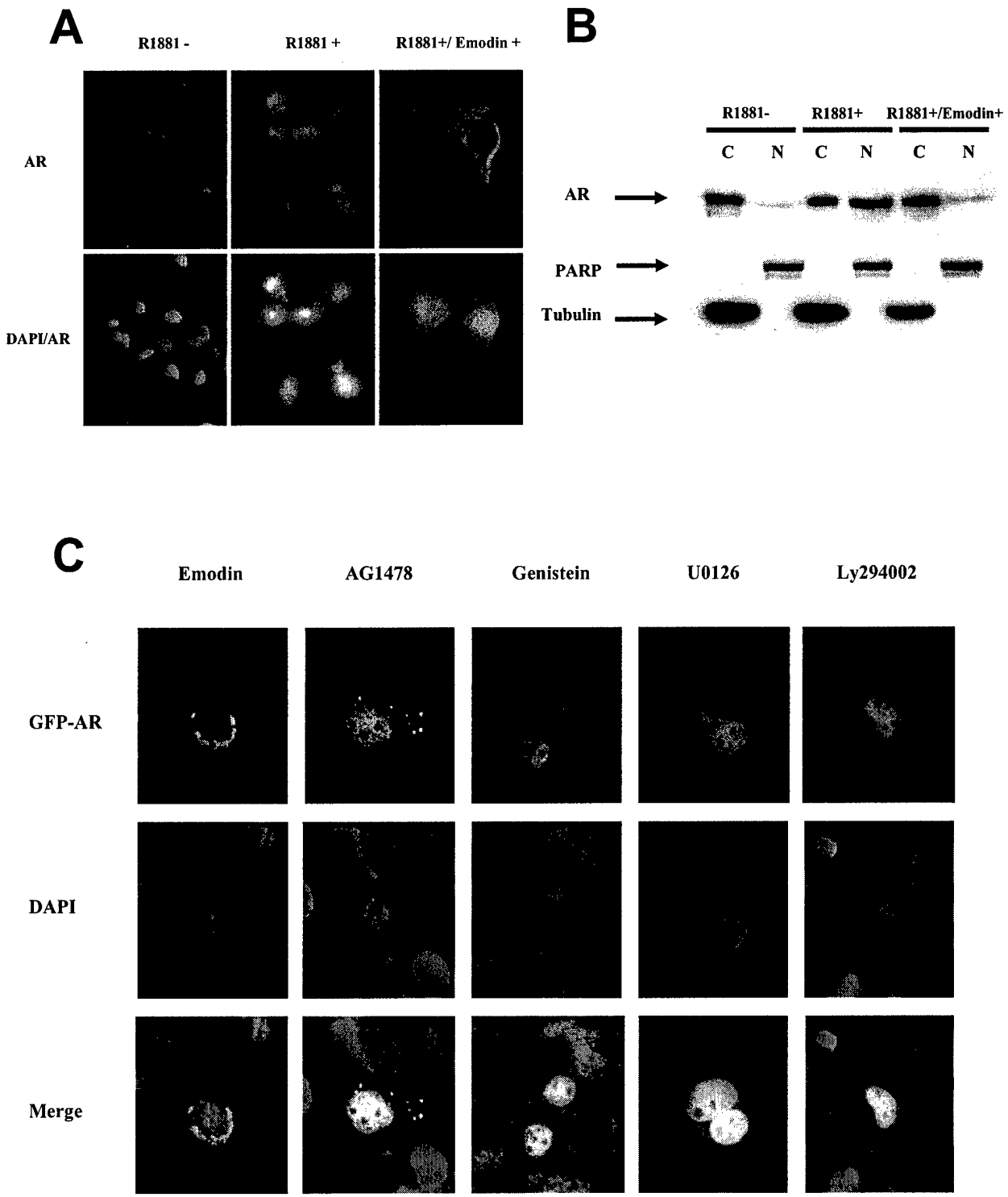

A
R1881 -
R1881 +
R1881+/ Emodin +
AR
DAPI/AR
B
R1881-
R1881+
R1881+/Emodin+
C
N
C
N
C
N
AR
PARP
Tubulin
C
Emodin
AG1478
Genistein
U0126
Ly294002
GFP-AR
DAPI
Merge

Figure 4

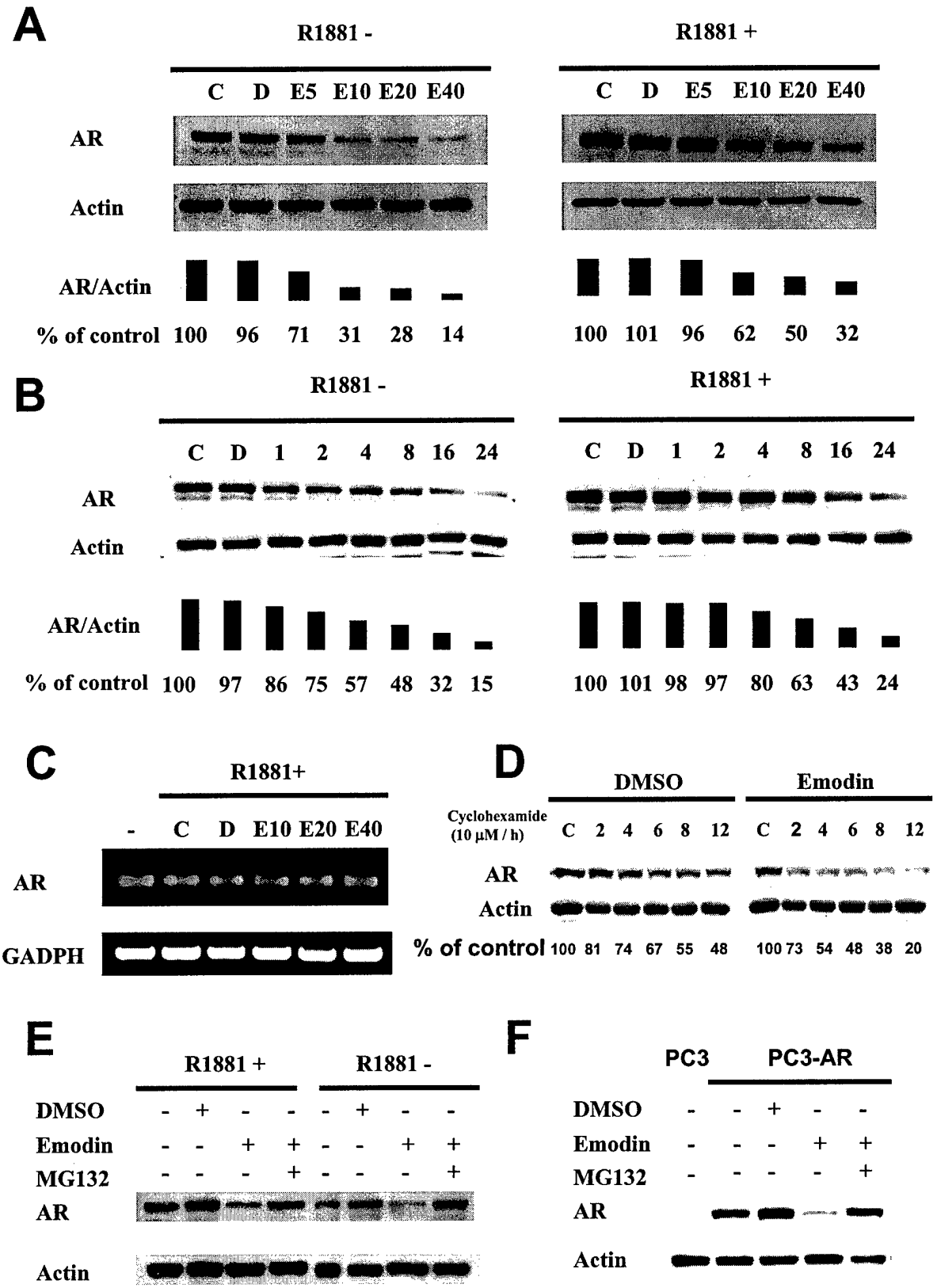

A
R1881 -
R1881 +
C D E5 E10 E20 E40
AR
Actin
AR/Actin
% of control 100 96 71 31 28 14
100 101 96 62 50 32
B
R1881 -
R1881 +
C D 1 2 4 8 16 24
AR
Actin
AR/Actin
% of control 100 97 86 75 57 48 32 15
100 101 98 97 80 63 43 24
C
R1881+
- C D E10 E20 E40
AR
GADPH
D
DMSO
Emodin
Cyclohexamide (10 μM / h)
C 2 4 6 8 12
C 2 4 6 8 12
AR
Actin
% of control 100 81 74 67 55 48
100 73 54 48 38 20
E
R1881 +
R1881 -
DMSO - + - - - + - -
Emodin - - + + - - + +
MG132 - - - + - - - +
AR
Actin
F
PC3
PC3-AR
DMSO - - + - -
Emodin - - - + +
MG132 - - - - +
AR
Actin

Figure 5

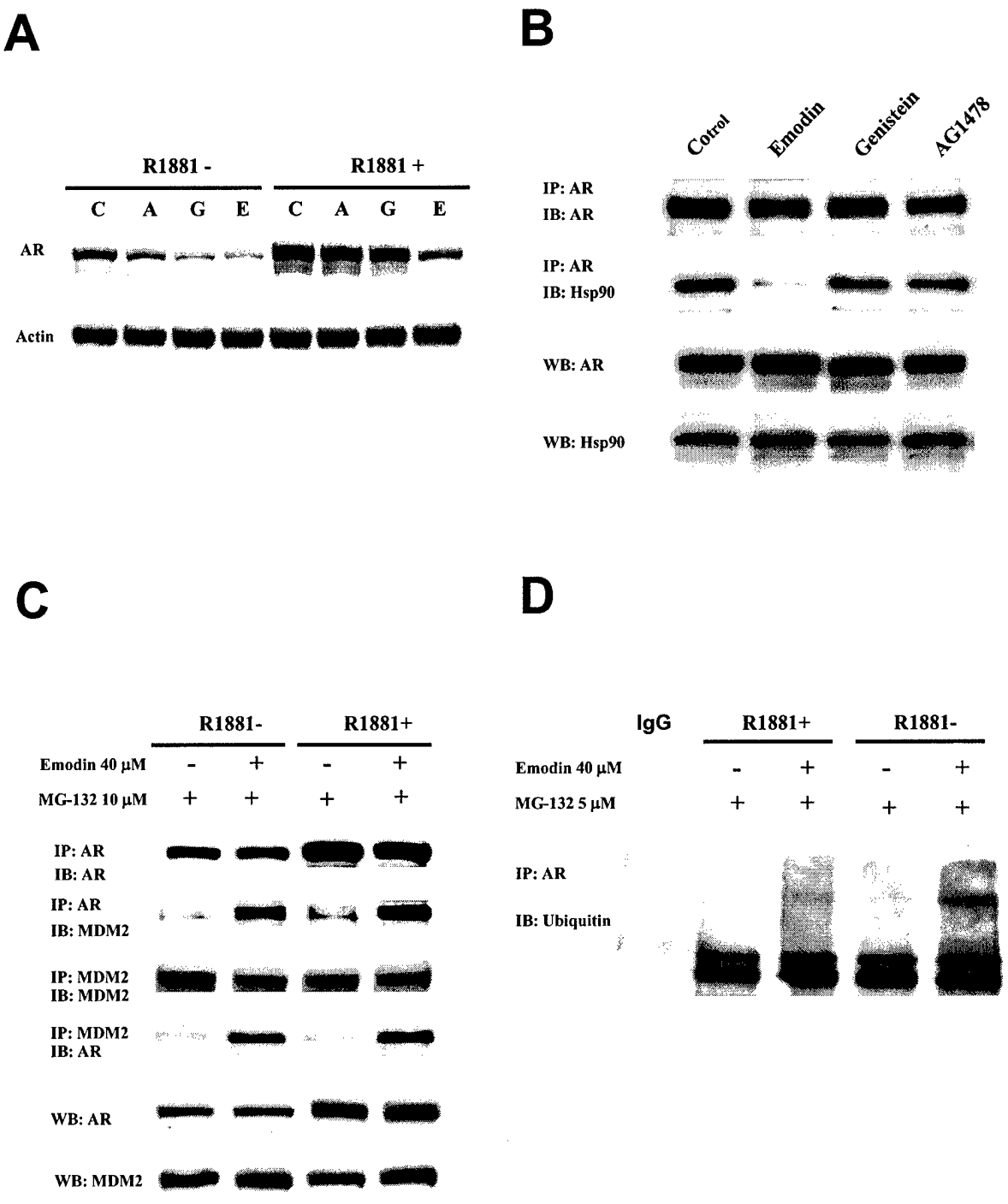

A
R1881 -
R1881 +
C A G E C A G E
AR
Actin
B
Control
Emodin
Genistein
AG1478
IP: AR
IB: AR
IP: AR
IB: Hsp90
WB: AR
WB: Hsp90
C
R1881-
R1881+
Emodin 40 μM - + - +
MG-132 10 μM + + + +
IP: AR
IB: AR
IP: AR
IB: MDM2
IP: MDM2
IB: MDM2
IP: MDM2
IB: AR
WB: AR
WB: MDM2
D
IgG
R1881+
R1881-
Emodin 40 μM - + - +
MG-132 5 μM + + + +
IP: AR
IB: Ubiquitin

Figure 6-1

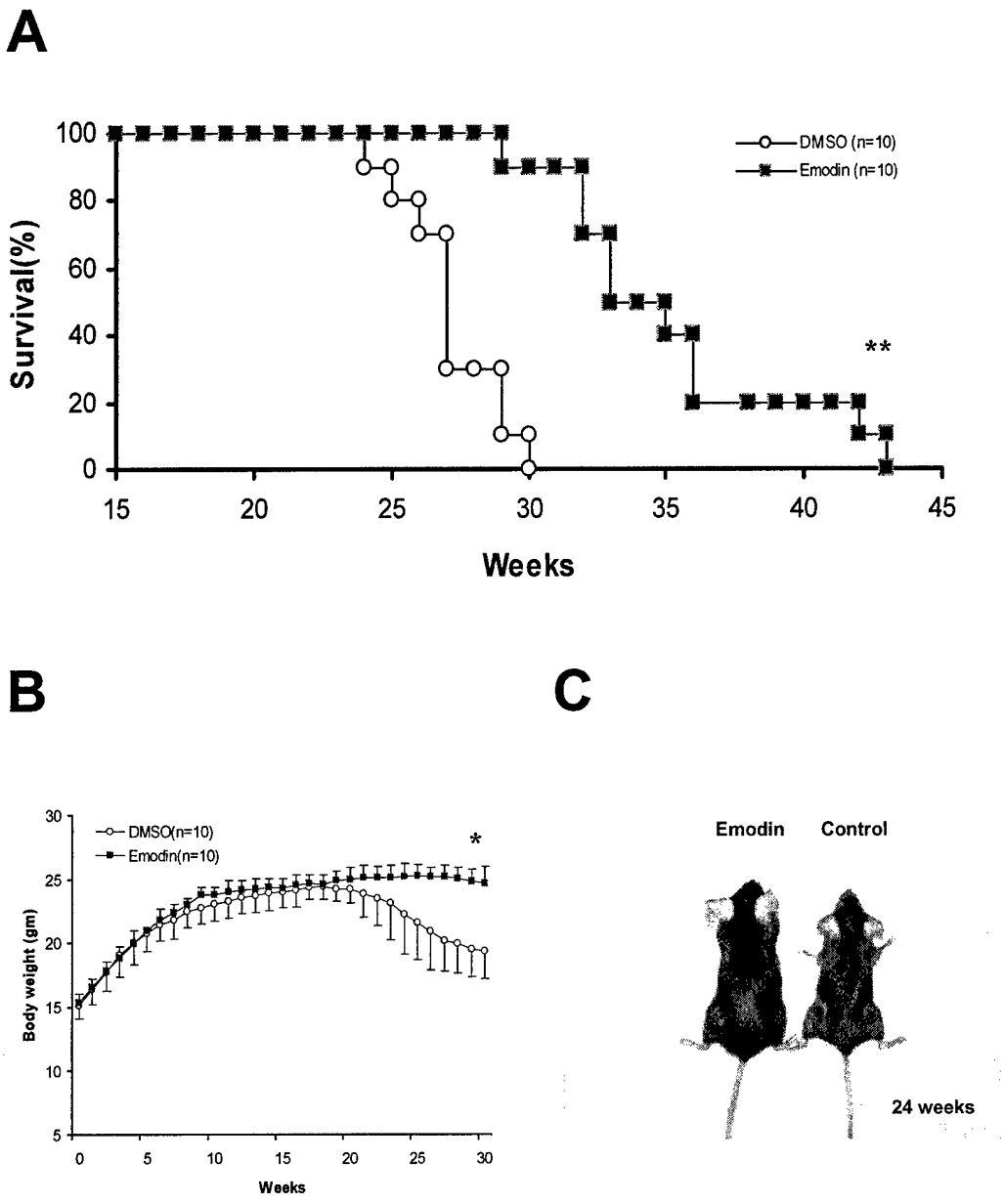
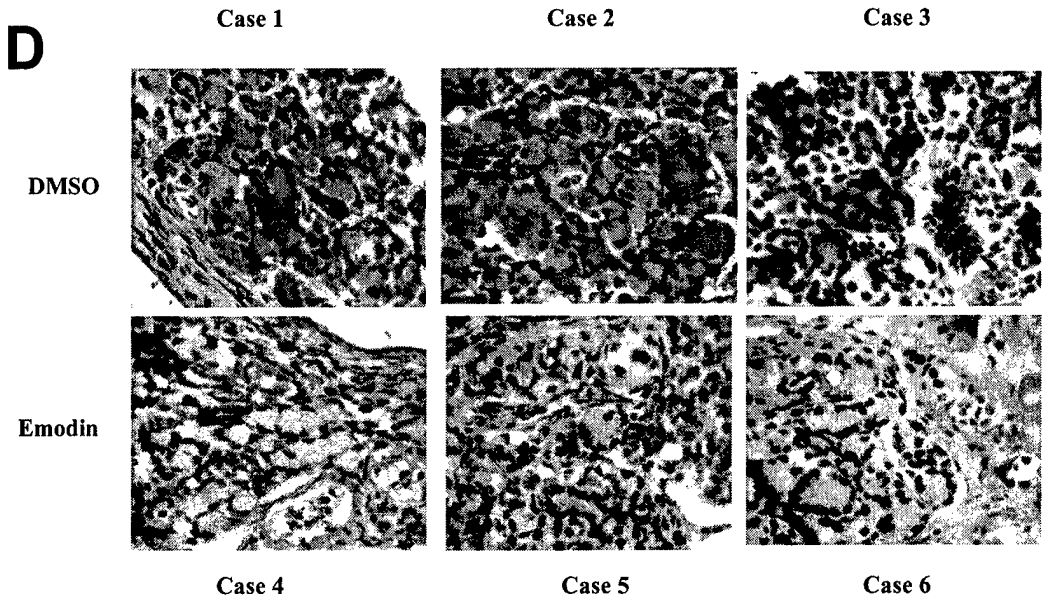
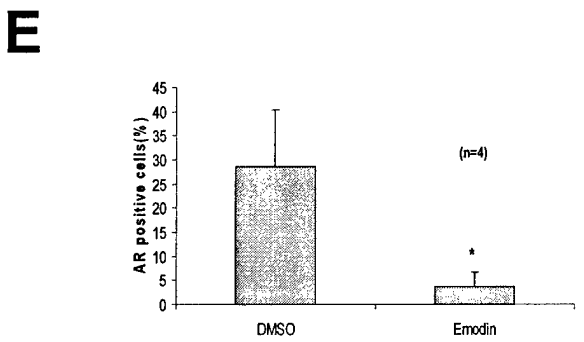
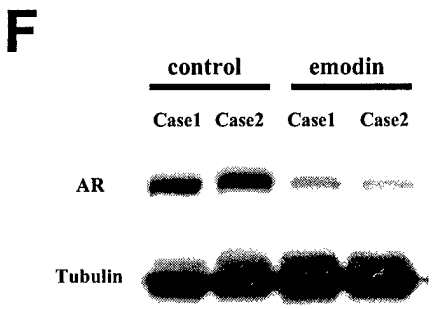
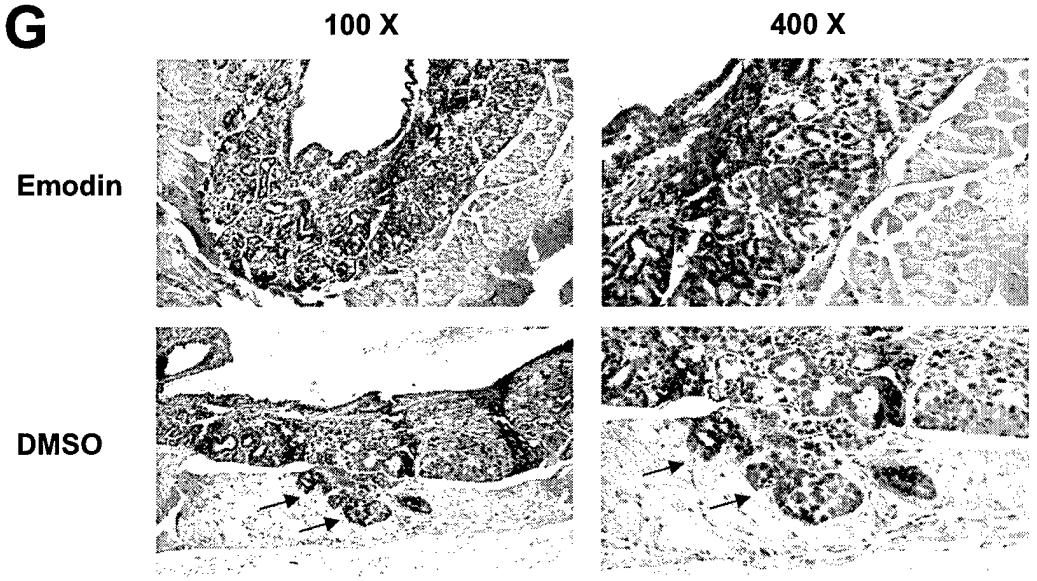

A
100
80
60
40
20
0
Survival(%)
15
20
25
30
35
40
45
Weeks
DMSO (n=10)
Emodin (n=10)
**
B
30
25
20
15
10
5
Body weight (gm)
0
5
10
15
20
25
30
Weeks
DMSO(n=10)
Emodin(n=10)
*
C
Emodin
Control
24 weeks

Figure 6-2


D
Case 1
Case 2
Case 3
DMSO
Emodin
Case 4
Case 5
Case 6


E
AR positive cells(%)
45
40
35
30
25
20
15
10
5
0
(n=4)
*
DMSO
Emodin


F
control
emodin
Case1
Case2
Case1
Case2
AR
Tubulin


G
100 X
400 X
Emodin
DMSO